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OBSERVATIONS ON SOME HISTOCHEMICAL REACTIONS IN THE HUMAN PLACENTA, WITH SPECIAL REFERENCE TO THE SIGNIFICANCE OF THE LIPOIDS, GLYCOGEN AND IRON

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SEVERAL convergent events in the past few years have focused attention upon some of the chemical and physiological mechanisms in the placenta. It is now well known that estrogens and progesterone are present in the placenta, and it is generally believed that these substances are elaborated as well as stored there. Moreover, studies of the transfer of material across the placenta have led to the necessity of postulating mechanisms capable of providing energy for the transfer processes, and to the question of the localization of these mechanisms. Finally, a detailed cytological study (Wislocki and Bennett, 1943) has formed a sound morphological basis for the investigation of certain histochemical reactions and their localization in the human placenta.

MATERIAL AND METHODS

The principal material employed in the present study consisted of nine human placentae secured at operation or at term.[2] The youngest placenta was from an embryo of 3 mm. in length. Three specimens of approximately six weeks of age were also available. In addition, a three-months' placenta of a rhesus monkey (Macaca mulatta) was studied with the same procedures, and, since no significant differences were observed between it and the human material, it has been included in the present report. Parts of the specimens

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were fixed as soon as possible in various reagents for special cytological reactions, while other parts were used for determining the reactions of fresh, unfixed tissue.

Fluorescence studies were made on fresh and formalin-fixed specimens, employing transillumination of the tissues with ultraviolet rays derived by filtering the light from an iron-carbon arc through a Corex 586 filter and a copper sulfate solution. The ultraviolet light was concentrated by quartz collimating and condensing lenses. Observations on the birefringence of fresh and formalin-fixed placentae were made with a Spencer polarizing microscope. Microincinerated specimens were prepared after fixation in absolute alcohol and formalin, as recommended by Scott (1933), and were observed by darkfield illumination. The ashed sections were especially useful for the localization of iron, since its oxides are characterized by a yellow or orange color. For comparison, iron was also detected by the Turnbull blue reaction on sections of tissues fixed in formalin or in alcohol-formalin.

The Schiff, or "plasmal" reaction of Feulgen and Voit (1924), was utilized for the study of aldehyde-like compounds. The procedure was applied both to fresh and formalin-fixed tissues, as recommended by Lison (1936). Similarly, strong sulfuric acid (Liebermann-Burchardt reaction) was applied to fresh and formalin-fixed tissues in an effort to determine the localization of unsaturated steroid-like substances (Dempsey and Bassett, 1943). Glycogen was observed by staining with Best's carmine or by the Bauer method after fixation in absolute alcohol, formalin and picric acid.

The oxidative systems of the placenta were studied by the application of the "nadi" reagents (paraphenylene-diamine and alpha-naphthol), and by determining the ability of the various placental regions to oxidize or reduce a series of reduction-oxidation indicator dyes. Dilute solutions of these dyes and reagents were prepared in a phosphate buffer at pH 7.4, and were applied to spreads of fresh, unfixed placental tissue.

OBSERVATIONS

Fluorescence

The fluorescence of a variety of tissues has been described by Hamperl (1934), who showed that both the color and intensity of fluorescence varied greatly in different structures. Moreover, a number of studies have indicated that tissue fluorescence may be used as an analytical tool. Bierry and Gouzon (1936) showed that purified estrogenic substances fluoresced with characteristic emission bands in the green, yellow and red regions of the spectrum. Popper and Greenberg (1941) and Ragins and Popper (1942) have described a fading, green fluorescence in a number of animal tissues, a color which they consider identical to that of vitamin A. Yet, in studying the fluorescence of ovarian tissue, Dempsey and Bassett (1943) observed a lipoid soluble, stable fluorescence in the green and yellow regions of the spectrum which was associated with the thecal, luteal and interstitial tissue. Because of the similarity in color to that observed by Bierry and Gouzon and because of the anatomical localization in tissues which give other reactions for steroid substances, Dempsey and

Bassett regarded the ovarian steroids as a likely source of this fluorescence.

Unfixed spreads of fresh tissue and frozen sections from fresh and formalin-fixed placentae were examined with the fluorescence microscope. The outermost layer of the placental villi fluoresced with an intensity greater than that of any other region. The color of the emitted light ranged, in different regions, from a whitish-green to a greenish-yellow. The central core of the villus was in sharp contrast to this outer greenish zone by virtue of its dim, bluish-lavender fluorescence. This lavender core appeared to occupy the entire center of the villi except for the spaces filled by the fetal blood vessels, which were revealed by the weak, muddy brown fluorescence of the fetal blood contained within them. The outer region of intense, greenish fluorescence followed accurately the distribution of the syncytial trophoblast. This outer rim of greenish fluorescence was present in all of the placentae examined. In the later stages of gestation, the progressive thinning of the syncytium was reflected in the similar narrowing of the green fluorescent margin. Even in term placentae, however, this outer layer of the villus retained its characteristic intense fluorescence. The intensity of the light emitted by this layer was uniform except for the spaces occupied by the nuclei, which did not fluoresce and therefore appeared dark. The greenish fluorescence was extremely intense in the buds of syncytium which frequently project from the surface of villi. The inner core of weaker, lavender fluorescence was associated with the fetal connective tissue which makes up the stroma of the villi. Between the inner lavender region and the outer greenish border a somewhat indistinct intermediate zone sometimes occurred. The intermediate zone was only detected in sections from early placentae, and, even in them, was not invariably present. The color of the light emitted by this irregular narrow band was greenish, similar to that of the syncytium, but the intensity was very weak compared to that of the outer layer. In a few favorable sections, this inconstant intermediate zone appeared to be associated with the presence of Langhans' cells.

Spreads and sections containing cytotrophoblastic cell columns were also examined for fluorescence. The cytoplasm of the cellular trophoblast of the cell columns emitted greenish light of a very dim intensity, similar in all respects to that given off by the cells of the Langhans layer.

The solubility characteristics of the substances giving the greenish fluorescence to the syncytial layer were tested with a number of solvents. The greenish fluorescence was present in fresh and formalin-fixed villi. It was present but decreased in intensity in villi which had been extracted for 24 hours or more with normal saline, $\frac{2}{3}$ saturated ammonium sulfate or formamide. It was greatly decreased or abolished in sections extracted with acetone or alcohol.

Birefringence

Although definitive conclusions regarding the nature of lipoidal substances cannot be drawn from data on birefringence alone, the behavior of lipoids when examined with polarized light can nevertheless yield information of considerable value when combined with observations made by other methods. Cholesterol, its derivatives and their esters, frequently form birefringent crystals, and also occur as spherocrystals in the fresh state (Lison, 1936). Consequently, the observation in tissues of lipoidal crystals which are birefringent, and especially of lipoidal droplets which show the black cross of polarization, is in harmony with the expected behavior of cholesterol derivatives.

Fresh, teased villi were mounted in normal saline or in glycerol and examined immediately with the polarizing microscope. The villi from the younger placentae contained lipoidal droplets which were easily resolvable with the 45 X objective. These droplets were invariably present in the syncytial trophoblast lining the villi as well as in the buds of pure syncytium which frequently project from the villi. In most of the villi examined the lipoidal droplets were uniform in size, although there were occasional villi in which droplets larger than the rule were encountered. The droplets were birefringent and showed the Maltese cross of polarization when examined between crossed prisms. Figure 5 illustrates these anisotropic lipoids of the syncytium. The stroma of the villi contained connective tissue fibers which were also birefringent, but which were arranged parallel to each other for the most part so that complete extinction of these elements could be secured by rotation of the stage. Similarly, the brush border which occurs in parts of the free margin of the syncytium was birefringent. Apparently this border is composed of fibrillar elements arranged in parallel because its birefringence could be completely extinguished by rotation of the stage.

Villi from older placentae, including those secured at term, also contained anisotropic lipoidal droplets localized in the syncytium. These droplets were considerably smaller than in the younger specimens. In the term placentae these fine, dust-like, birefringent particles were so small that they were nearly unresolvable with the high-dry objective.

Birefringence of the syncytium was also observed in villi teased on a slide after fixation in formalin, and in frozen sections made from both fresh and formalin-fixed specimens. The double refraction was never as brilliant in these preparations as it was in the fresh, teased material. Nevertheless, the sectioned villi were a useful adjunct since more accurate localization of the anisotropic lipoids of the syncytium was possible than in the teased preparations.

The birefringent substances of the syncytium were studied after immersion of the villi in a number of solvents. Acetone removed the

doubly refractile material completely. Prolonged immersion in saline solutions caused a diminution in the amount of birefringence, as did also extraction with $\frac{2}{3}$ saturated ammonium sulfate. On the other hand, the fibrillar double refraction associated with the stroma and the brush border of the syncytium was not appreciably altered by these procedures.

Sulfuric acid reaction

Treatment of sterol substances with concentrated sulfuric acid, together with other reagents, produces a play of colors which has been utilized in a number of chemical and histochemical tests such as the Salkowski and the Liebermann-Burchardt reactions. These procedures involving the use of strong sulfuric acid were formerly thought to be specific for cholesterol (Romeis, 1928), but, more recently, systematic study of the reactions has shown them to indicate unsaturation in sterol molecules (Fieser, 1937; Sobotka, 1938), and a large number of naturally-occurring sterols which give positive reactions are now known (Reichstein and Shoppee, 1943). Moreover, the application of strong sulfuric acid to sections of the ovary caused a play of colors localized in the same regions in which other steroid reactions were produced (Dempsey and Bassett, 1943). It appears, therefore, that the sulfuric acid reaction is one which depends upon unsaturation in steroid molecules, and that, when applied to sections, it yields information in agreement with that obtained with other sterol reactions.

In a number of instances sulfuric acid was applied to frozen sections or teased preparations of both fresh and fixed placentae. The reaction was negative or only weakly positive in the fresh material. On the other hand, immediately following the application of the acid to preparations fixed in formalin, a faint reddish color developed which gradually darkened to a final greenish brown. Microscopical examination revealed that the development of color occurred in the syncytial layer (Fig. 1). At the magnifications employed (600 X), the color was distributed throughout the syncytial cytoplasm rather than agglomerated into droplets as was the case in similar observations on the ovary (Dempsey and Bassett, 1943).

The Schiff reaction

The recolorization of leucopararosaniline has long been used as a test for aldehydes (Schiff, 1866). The test is by no means specific for aldehydes, however, since certain ketones and other unsaturated substances also give positive reactions (Lison, 1932). Moreover, Feulgen and Voit (1924) demonstrated that a fat-soluble substance which they called "plasmalogen" was present in the adrenal cortex and corpus luteum, which, after treatment with mild oxidizing agents, gave a positive Schiff reaction. The identification of this substance has not been definitely accomplished. Feulgen, Imhauser and Behrens (1929)

suggested palmitic and stearic acids as a possibility, and Verne (1929) oleic acid, although the latter author was unable to oxidize these substances with oxidizing agents as weak as those which are effective when applied to tissues.

Whatever the nature of the substance or substances responsible for the Schiff or "plasmal" reaction, it is apparent from the observations cited above that the reaction discloses certain fat-soluble materials in organs which have an active sterol metabolism. Consequently, it was thought of interest to determine if such a substance was present in the placenta.

Fresh and formalin-fixed villi were placed in Schiff's reagent following immersion in one per cent mercuric chloride. An immediate reaction occurred, as indicated by the development of a violet color in the outer layer of the villi. The reaction was negative in tissue which was previously extracted with alcohol or acetone, and in tissue which was not treated with the mercuric chloride. Prolonged washing in water for 24 hours did not prevent the reaction in the formol-fixed tissues.

Frozen sections were also prepared. The sections were treated on the slide with mercuric chloride and Schiff's reagent. Upon completion of the reaction the tissue was washed in an acid-metabisulfite solution, dehydrated in cellosolve and mounted in balsam after clearing in oil of origanum. Examination of sections prepared in this way revealed that the violet color was concentrated in the syncytium. The Langhans layer and the Hofbauer cells were weakly positive, while the stroma was entirely negative.

Indophenol oxidase reaction

The catalysis by tissues of the formation of indophenol from alpha-naphthol and p-phenylenediamine is commonly believed to reveal the presence of the cytochrome oxidase-cytochrome c system. Moreover, this system, because of its almost universal occurrence, and because of the marked decline in metabolism after cytochrome inhibition, is thought to provide one of the principal avenues for energy transfers in animal tissues. The observation, therefore, of the amount of indophenol blue produced allows a rough estimate of the amount of oxidative activity in a given region.

The application of the nadi reagents to fresh, unfixed, teased villi and unfixed frozen sections was followed by the production of indophenol blue in the syncytial layer (Fig. 4). The lipoidal droplets of the syncytium were easily visible in these preparations because of the concentration of indophenol at their peripheries. Moreover, in addition to the fat droplets, fine granules appeared in the syncytial cytoplasm. These granules were localized for the most part in the intermediate and basal regions of the syncytium, and occurred only rarely near the free margin. They were concentrated around the

peripheries of the nuclei which, in consequence, appeared to be outlined by a dark margin. The stroma, in contrast to the syncytial trophoblast, contained no perceptible amount of indophenol and, in consequence, remained unstained.

Reduction-oxidation indicators

The observations mentioned in the preceding section concerning the high concentration of indophenol oxidase in the syncytium but

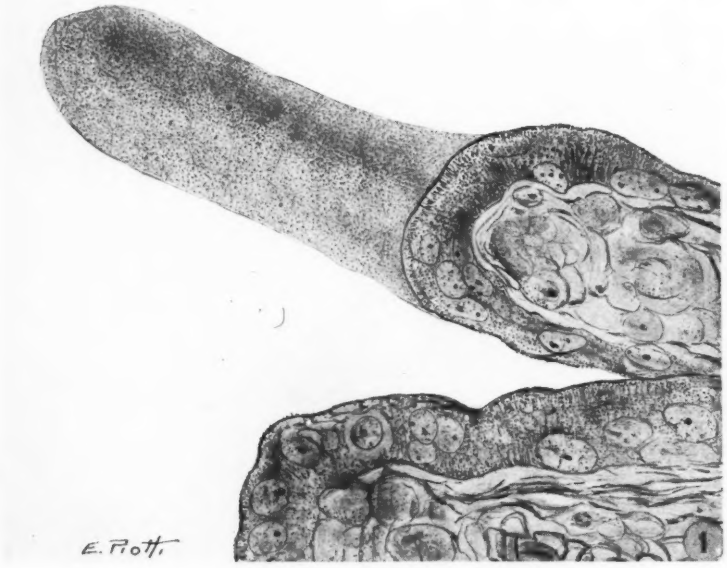

FIG. 1. Drawing of the sulfuric acid reaction in a chorionic villus from a human placenta of six weeks. The fine stippling, shown in the syncytial layer, represents the localization of the red to greenish-brown reaction. 40× objective, 10× ocular.

not in the stroma suggested that these regions might have quite different reduction-oxidation potentials, since the potentials of the cytochromes are all positive. Consequently, fresh, teased villi and fresh frozen sections of villi were stained with a series of reduction-oxidation indicators. The syncytium concentrated all of the dyes in their oxidized form. On the other hand, the stroma was unstained by dichlorphenol-indophenol and toluylene blue, while only the nuclei of the stromal cells stained with methylene blue and Lauth's violet. The cytoplasm of the Hofbauer cells stained deeply with methylene blue and Lauth's violet.

These observations indicate that the syncytial layer is maintained in air at a more positive redox potential than are the deeper parts of

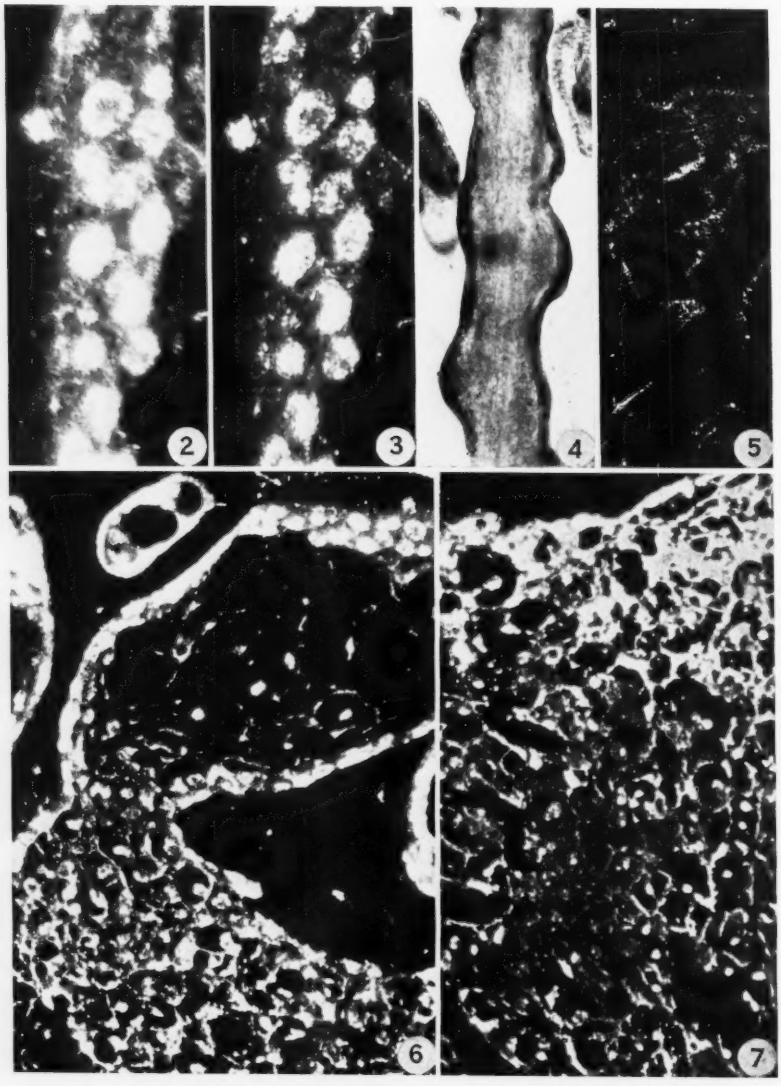

FIG. 2. Photograph of a microincinerated section through the syncytial trophoblast of a human chorionic villus. The embryo from this specimen had a crown-rump length of 2.5 cm. The section was photographed without any filter, and shows the total distribution of ash. ×775.

FIG. 3. The same field illustrated in fig. 2, photographed through a red (Wratten A) filter. The photograph registers the red or yellow light caused by the presence of iron. ×775.

FIG. 4. Indophenol oxidase reaction in a human chorionic villus of the 6th week of pregnancy. The oxidase activity is confined to the syncytial trophoblast. ×100.

the villus. The observations are in keeping with the similar distribution of indophenol oxidase, and may be a result of a predominant effect of the cytochrome system upon the expressed potential of the syncytium.

Glycogen

Since the discovery by Claude Bernard (1879) that the carbohydrate stores of the animal organism are mainly glycogen, numerous studies on the distribution of this substance have been carried out (for references, see Needham, 1931, 1942). The principal results of these investigations can perhaps be stated by the generalizations that stainable glycogen enjoys a much wider distribution in embryonic than in adult organs and tissues, and that, in the adult, glycogen is usually present in tissues which are poorly vascularized and metabolically sluggish. The converse of this latter statement does not necessarily follow, however, since glycogen is readily demonstrable in some active tissues, notably the liver.

It is well known that glycogen is present in the placenta. There is, however, some disagreement as to its localization among the various placental tissues. Windle (1940) states that glycogen is found only in the maternal placenta. This statement appears to be true for the mouse (Driessen, 1908) and rabbit (Chipman, 1902; Driessen, 1908). On the other hand, the rat placenta, according to Goldmann (1912), contains some glycogen in the syncytium. In man, glycogen is readily demonstrable in the fetal elements comprising the cytotrophoblastic shell and cell columns, whereas none, or at most very faint traces, is found in the syncytium and the Langhans cells (Driessen, 1907, 1908; Flesch, 1911; and Wislocki and Bennett, 1943).

In several human placentae which we have examined for glycogen in the course of the present study, we have found abundant stores of it in the cytotrophoblastic cell columns and shell. In the cell columns it occurs in mere traces in the most proximal portions of the columns where they attach to the secondary villi, but as one

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FIG. 5. Photograph of the birefringent lipoids in the syncytium of a monkey's placenta. A fresh spread of the placenta was photographed between crossed prisms. ×80.

FIG. 6. Microincinerated section through a chorionic villus and cytotrophoblastic cell column from the same placenta illustrated in fig. 2. The dense deposits of ash in the matrix surrounding the cytotrophoblastic cells have a yellow color when observed under the microscope, denoting the presence of iron. Compare with fig. 10. Note also the dense concentration of ash in the syncytium clothing the chorionic villus. ×280.

FIG. 7. Microincinerated section through the cytotrophoblastic shell and decidua from the same placenta illustrated in figs. 2 and 6. The extremely dense ash associated with the cytotrophoblastic shell can be seen at the top of the figure. To the eye, when observed through the microscope, this ash has a yellow color. The decidual cells, in the lower two-thirds of the figure, contain a more evenly distributed bluish ash interspersed with occasional yellow particles and exhibit a much less pronounced pericellular condensation than that associated with the cytotrophbolastic elements. Compare with fig. 11. ×280.

follows the columns proximally toward their attachment to the trophoblastic shell, the cells gradually become heavily laden with glycogen. The trophoblastic cell islands contain similar deposits of glycogen. The Langhans cells and the syncytium of the secondary villi are devoid of glycogen. The mesenchymal connective tissue stroma forming the closing plate of the fetal placenta, in which the umbilical vessels branch to form the large placental blood vessels, also contains appreciable amounts of glycogen. These deposits can be perceived in dwindling amounts in the stroma of the major stem villi which penetrate the placenta. In the delicate stroma of the great profusion of terminal secondary villi, only traces of glycogen can occasionally be identified.

The maternal tissues, beneath the fetal placenta, are rich in glycogen. In placentae of the first months of pregnancy the epithelial cells of the uterine glands are excessively rich in glycogen. In the decidual cells glycogen is also readily demonstrable, although with considerable variability as to individual cells, and diminishing as gestation advances.

The distribution of glycogen in the fetal portions of the human placenta is, in general, in agreement with the thesis expressed above that glycogen accumulates in poorly vascularized and metabolically sluggish regions, since the regions known to be rich in glycogen, namely, the cytotrophoblastic shell and cell columns, are not directly supplied with fetal blood vessels nor directly bathed by the maternal circulation. Similarly, the maternal placenta (decidua), which is also rich in glycogen, is very poorly vascularized. Conversely, the syncytium and Langhans layer contain only small amounts of glycogen and are in close approximation to both the fetal and maternal blood streams.

Microincineration and iron reactions

Inorganic mineral salts may be detected and localized within tissues by the method of microincineration (Policard, 1923; Scott, 1933). The procedure involves heating sections to a temperature sufficiently high for the complete combustion of all organic material. Unfortunately, the total mineral content cannot be determined because some mineral elements are volatilized at the temperatures employed, nor, except for iron, can individual elements be easily identified. However, differences in the mineral ash concentration in cells and tissues can easily be determined by observation of the incinerated specimens with dark-field illumination. Moreover, iron alone of all the biologically occurring elements can easily be recognized by virtue of the reddish or yellowish color of its oxide.

Observation of microincinerated specimens of human placentae revealed that the greatest concentration of ash occurred in the syncytial trophoblast. The distribution of ash in the cytoplasm of the syncytium was regular and even, except for the region of the nuclear

membrane in which a heavier condensation was observed than anywhere else (Figs. 2 and 6). The outer margins of the syncytium showed different appearances in different areas. In some villi, it was smooth and consisted of a condensation of ash slightly greater than that of the subjacent cytoplasm. In other areas this marginal condensation was entirely lacking, in which event the outer surface of the syncytium was irregular, and delicate processes of stippled ash deposits were seen projecting into the intervillous space.

The relative amount and distribution of the iron ash in the placenta can be readily told and distinguished from the non-iron containing ash by photographic means. Since the iron residues are yellowish or orange in color, while all other ash is bluish, it is possible to effect a separation of the iron by means of a suitably colored filter. Figures 2 and 3 are photographs of the same region made without and with recourse to a red filter. In the former, the total ash is registered, while in the latter, only the red or yellow light cast by the iron-containing residues passed the filter. Consequently, only the iron residues are registered in the photograph.

The incinerated syncytium was, for the most part, blue or bluish-white in color. When examined with the higher power objectives, however, yellow or orange ash having a quite definite distribution could be detected. The yellow ash, which, according to Policard (1923) and Scott (1933b) reveals the presence of iron, was localized in a thin but dense shell surrounding the nuclei of the syncytium and as fine granules scattered diffusely and in low concentration throughout the syncytial cytoplasm. In some of the villi the outer margin of the syncytium contained considerably greater quantities of iron than did adjacent regions.

The cytotrophoblastic cells of the Langhans layer, the cell columns and the cytotrophoblastic shell were all similar to one another in appearance and could be distinguished easily from the syncytium. The outstanding characteristic of the cytotrophoblast was its predominantly yellow or orange color, caused by a relatively dense condensation of yellow ash in the intercellular matrix and by a similar but less dense condensation of yellow ash in the perinuclear region. The cytoplasm of the cytotrophoblastic cells also contained a small amount of yellow or orange ash, scattered as small granules in a ground substance of bluish-white ash which was evenly distributed throughout the cytoplasm. This ground material of bluish ash was much less dense than it was in the syncytial cytoplasm.

The cytotrophoblastic cells could also be distinguished from the decidual cells by the distribution of ash (Fig. 7). Although both elements contained about the same amounts of bluish-white cytoplasmic ash, the conspicuous yellow pericellular and perinuclear deposits typical of the cytotrophoblast were absent in the decidua. The margins of the decidual cells were slightly denser than was the rest of the cytoplasm, but contained only occasional flecks of yellow ash.

A similar distribution of iron was detected in sections stained by the Turnbull blue reaction. In early placentae, considerable amounts of iron were observed in the syncytium (Fig. 9), whereas in older villi decreasing amounts of iron were present. The principal depot of iron encountered in older villi proved to be in the stroma immediately beneath the trophoblast, a finding which coincides with the previous observation of Hofbauer (1905).

The Langhans cells contained little demonstrable iron by the Turnbull blue method (Fig. 9). Similarly the cytotrophoblastic cells of the cell columns, cell islands and trophoblastic shell contained very little intracytoplasmic iron, although the prominent intercellular ground substance associated with the cytotrophoblast in these localities exhibited a very dense concentration of iron (Figs. 10 and 11). In the junctional zone the decidua could be fairly well distinguished from the cytotrophoblast by virtue of their respective reactions. The cytotrophoblast showed a dense concentration of iron in the peculiar ground substance associated with the typically granular and foamy trophoblastic cells, whereas the decidual cells were surrounded by a delicate mesh of reticulum and interstitial fluid which stained only faintly.

DISCUSSION

The nature of the placental lipoids

The first four sections of this study deal with observations which bear upon the nature of the placental lipoids. The fat of the fetal placenta is localized almost entirely in the syncytial trophoblast, and is easily revealed in spreads of fresh material by its difference in refractility from that of the surrounding cytoplasm. It may also be demonstrated with ease by staining with Sudan III or osmic acid (Wislocki and Bennett, 1943). It is readily extractable by immersion of the tissue in alcohol or acetone. The lipoidal droplets may exist

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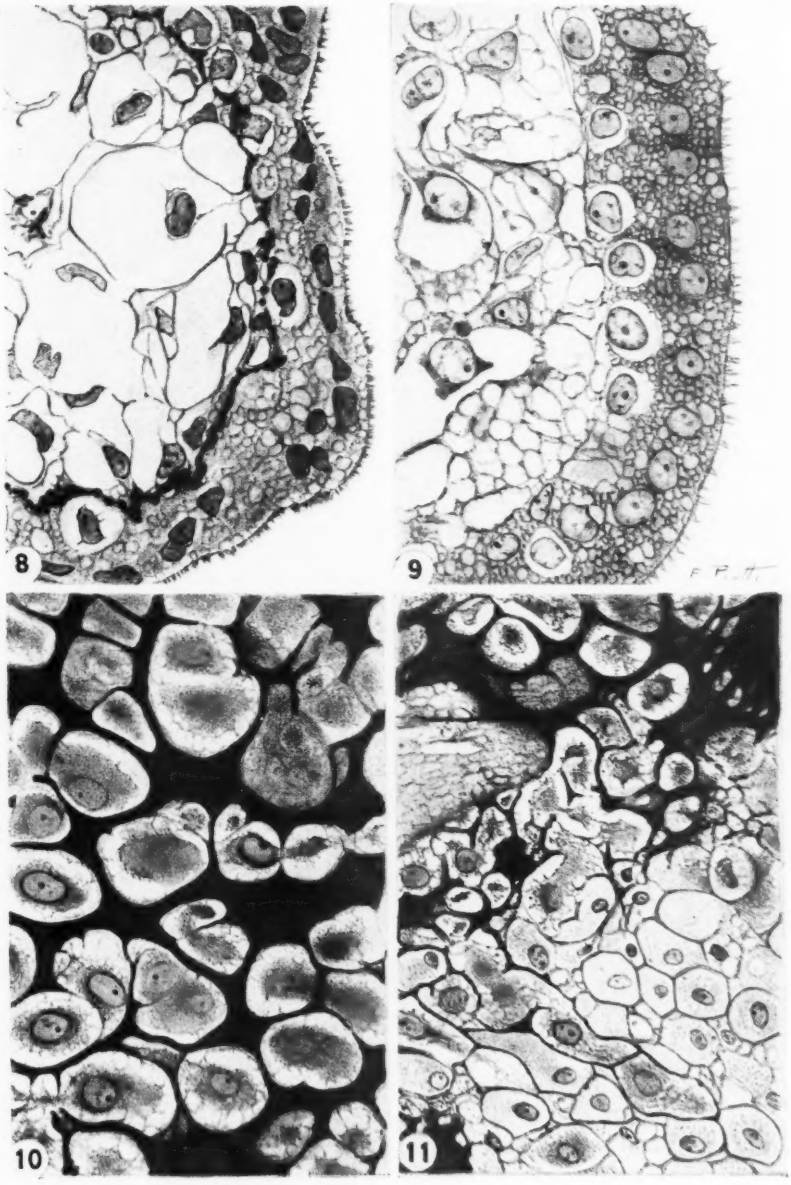
FIG. 8. Section through a chorionic villus of a human placenta of six weeks. The iron reaction consists mainly of particles localized in the stroma beneath the trophoblast, and occasionally present in the basal part of the syncytium. 40× objective, 10× ocular.

FIG. 9. Section through a human chorionic villus associated with a 3 mm. embryo. The iron reaction is readily demonstrable throughout the syncytical trophoblast. Little or no reaction for iron is present in the Langhans cells. 90× objective, 7× ocular.

FIG. 10. Section through a cytotrophoblastic cell island from a human placenta of six weeks. An intense iron reaction is demonstrated in the intercellular matrix. 90×objective, 7×ocular.

FIG. 11. Junctional zone from a human placenta of six weeks. An intense iron reaction is demonstrated in the intercellular matrix of the cytotrophoblastic shell. The cytoplasm of the cytotrophoblastic cells exhibits scarcely any blue. The pale decidual cells (at the lower right) have a much more delicately blue staining intercellular substance than do the cytotrophoblastic cells and in contradistinction to the cytotrophoblast the cytoplasm of the decidual cells shows a very faint stippling of blue. 40×objective, 7×ocular.



The figures on this plate are drawings representing iron reactions carried out with the Turnbull blue method. Basic fuchsin was used as a counterstain, although in the drawings the only color represented is that associated with the Turnbull blue reaction.

as birefringent spherocrystals showing the Maltese cross of polarization, or, under certain conditions, they may solidify into fine, dust-like birefringent crystals oriented at random in the plane of the section. The fatty material gives rise to a characteristic greenish-yellow or greenish-white fluorescence. Lastly, the syncytial lipoids form yellow phenylhydrazones from phenylhydrazine (Wislocki and Bennett, 1943), react to concentrated sulfuric acid with a play of colors, and give a positive plasmal reaction with Schiff's leucofuchsin reagent.

The reactions, cited in the preceding paragraph, are in accord with the thesis that the placental lipoids contain appreciable amounts of steroidal compounds. It is well known that cholesterol and related substances form birefringent droplets and crystals (Lison, 1936). Ultraviolet irradiation of pure steroidal substances causes fluorescence in the green and yellow regions of the spectrum (Bierry and Gouzon, 1936). The Liebermann-Burchardt reaction is positive in unsaturated steroids (Fieser, 1937; Sobotka, 1938; Reichstein and Shoppee, 1943). The phenylhydrazine reaction which reveals a carbonyl grouping is easily produced in the adrenal (Bennett, 1940), testis (Pollock, 1942), and ovary (Dempsey and Bassett, 1943), as well as by pure crystalline ketosteroids (Bennett, loc. cit.).

The phenylhydrazine reaction has been criticized as a test for ketosteroids by Gomori (1942). He pointed out that the reaction required treatment of the tissues by oxidizing agents, and that it gave results identical with those of the plasmal reaction of Feulgen and Voit (1924) and Verne (1929). This reaction consists of the recolorization of Schiff's reagent, leucofuchsin, after previous exposure of the tissues to mild oxidizing agents. Schiff's reaction is commonly believed to be an aldehyde reaction, and consequently Feulgen (1924), Verne (1929), and Gomori (1942) regard it as probably indicating the presence of palmitic, stearic or oleic aldehydes, although Verne (1929) was unable to produce oleic aldehyde by oxidations as mild as those effective in tissues. However, the reagent is by no means specific for the aldehyde grouping. Lison (1932), in studying a large number of organic compounds, found that positive reactions were given by aldehydes, certain ketones, and even by some unsaturated compounds which contained no carbonyl groups of any kind. Indeed, the only common characteristic of the compounds giving positive reactions was the fact that all were unsaturated. Consequently, since unsaturated ketones give positive plasmal reactions, and since no naturally occurring lipoidal aldehyde is known to exist in the tissues which give plasmal reactions (Verne, 1937), Gomori's view that the phenylhydrazine and plasmal reactions are indicative of aldehydes and not ketones seems unsound. On the other hand, the occurrence of the plasmal reaction in the same areas in which steroid reactions depending upon characteristics other than the carbonyl grouping, such as fluorescence, birefringence and the sulfuric acid reaction, seems rather to

indicate that, despite its lack of specificity, the plasmal reaction in these particular cases may well be caused by the presence of unsaturated ketosteroids.

The necessity of oxidizing certain substances in order to unmask the plasmal and phenylhydrazine reactions, as emphasized by Gomori (1942), raises interesting questions as to the nature of the reactive lipoids as they exist in the tissues. A number of possibilities seem evident. Feulgen and Voit (1924) and Verne (1929) suggested that the oxidative treatment might transform organic acids into their corresponding aldehydes. This view now seems unlikely, since Verne (1929) was unable to produce oleic aldehyde by oxidative agents as mild as those which were effective in tissues, and since the reactions, at least in the adrenal, corpus luteum and placenta, are associated with lipoids whose reactions are not characteristic of fatty acids. Moreover, since pure steroids are known to react with both phenylhydrazine (Bennett, 1940) and Schiff's reagent (Dempsey, unpublished observations), the alternative view that steroid substances may be among the reactive substances of the tissues now seems justified. Since the reaction requires oxidation for its unmasking, it seems necessary to assume that any one of the three following assumptions is permissible. The naturally occurring steroids may exist in a reduced state; or the tissue steroids may be combined by fragile bonds with other substances; or the reactions may be poisoned by easily oxidizable, fortuitously occurring compounds.

The role of glycogen in placental metabolism

From examination of our observations on the oxidative reactions in placental tissue and on the distribution of glycogen, the thought suggests itself that glycogen is present in the placental regions which have a sluggish oxidative metabolism and is absent in regions where oxidative processes are easily demonstrated. In the placenta, glycogen is absent in the syncytium and the Langhans layer. These tissues are located in close proximity to oxygenated blood. On the other hand, glycogen is abundantly present in the cytotrophoblastic cell columns and the cytotrophoblastic shell, and also in the stroma of the chorionic plate, and in these regions the tissue is avascular and remote from an oxygen supply. Moreover, the observations on the distribution of indophenol oxidase and of positive oxidation potentials show that oxidative activity is higher in the syncytium, which is poor in glycogen, than in the cytotrophoblast, which is rich in glycogen.

On considering the possible relationship, it has occurred to us that the negative correlation between the occurrence of histologically demonstrable glycogen and the oxidative activity of cells is by no means confined, as a special case, to the placenta. To the contrary, a similar situation is so frequently encountered in other tissues that a statement of the inverse relationship may be enunciated as a general rule.

For example, there are large amounts of histologically demonstrable glycogen in fetal tissues, which have poor or deficient mechanisms for aerobic glycolysis (Needham, 1931), while little glycogen is found in corresponding adult tissues in which complete systems for oxidative metabolism are encountered (Stotz, 1942). Likewise, in the adult, the principal regions in which glycogen can be demonstrated in abundance, such as cartilage and the outer layers of certain stratified epithelia, are those with a low oxygen tension consequent to their remoteness from a vascular bed.

Perhaps the most clear-cut examples of the inverse relationship between glycogen deposition and the activity of aerobic oxidative mechanisms are to be found in the developing kidney and chorioid plexuses. In the fetal metanephros, Flexner (1939) has shown that the cytochrome system is easily demonstrable in the nephric tubules but not in the collecting tubules. Contrariwise, it is a well-known fact that in the fetal kidney glycogen is abundantly present in the collecting tubules, while the nephrons contain little if any (Goldmann, 1912; Sundberg, 1924). Likewise, in the developing chorioid plexus of the pig embryo Flexner and Stiehler (1938) found that the indophenol oxidase activity and the reduction-oxidation potential increased sharply at about the 100th day of gestation. Previous cytological studies by Weed (1917) had already established the fact that glycogen is abundant in the chorioidal epithelium of early pig embryos, and that it decreases sharply in amount in embryos over 100 mm. crown-rump length and vanishes completely by the time the fetuses have attained lengths of more than 150 mm. Comparison of these data indicates that glycogen decreases and disappears from the chorioid plexuses at the time that Flexner and Stiehler observed the rise in oxidation potential.

The rich deposits of glycogen which are to be found in the uterine endometrium during its secretory phase and in the decidual cells of the maternal placenta or deciduomata are in keeping with the relative ischemia of these tissues. The cyclic deposits of glycogen occur at the end of the growth and proliferative activities of the uterus, at the time of the greatest height of the endometrium. According to Daron (1936), the outer portions of the endometrium in the rhesus monkey are supplied by coiled arteries which increase in length and tortuosity during the luteal phase of the cycle. Since these relatively long arteries give off few branches during their courses, but end rather abruptly in fountains of precapillary arterioles in the most superficial layer of the endometrium, it is clear that the regions beneath the superficial capillary bed become more and more ischemic as the cycle progresses. The deposition of glycogen in the uterine glands apparently occurs *pari passu* with the development of this ischemia.

The deposition of glycogen in the maternal placenta and in de-

ciduomata is associated with an ischemia of the decidual cells caused by a mechanism entirely similar to that of the endometrium of the cycle. The erosion and reorganization of the implantation site result in the loss of the superficial capillary bed, and the spiral arteries consequently open directly into the intervillous space as demonstrated in the human by Spanner (1936). Since these spiral arteries give off no terminal branches in their course through the pars compacta and outer two-thirds of the pars spongiosa, the capillary bed of the region is poor and the oxygen tension must therefore be low. A similar relative avascularity also occurs in deciduomata, causing, according to Rossman (1940), a characteristic sequence of glycogen deposition, fatty degeneration, and, finally, frank necrosis.

The examples cited in the preceding paragraphs form the basis of the generalization that glycogen deposits are abundant in tissues characterized by a low respiratory metabolism and, conversely, that an active aerobic mechanism precludes the formation of stainable glycogen. These conclusions suggest in turn that glycogen might be indicative of regions in which anerobic metabolism prevails. Yet, such a positive statement concerning anerobic metabolism cannot be made. The anerobic mechanism, requiring as it does the reduction of one molecule of glycogen for the oxidation of another, metabolizes from six to ten times as much glycogen as is needed for the production of the same amount of energy by the aerobic means (Burk, 1937). Consequently, it is conceivable that glycogen would be deposited in tissues either because the metabolism, and therefore the rate of utilization of glycogen, was low, or because an intense anerobic metabolism demands a large accumulation of glycogen for the purpose of maintaining the fermentative processes.

The view which is presently advanced concerning the relationship between glycogen deposition and respiratory metabolism bears a certain similarity to that of Creighton (1896). This investigator, long before the era of respiratory enzymes, noted that glycogen was usually deposited in poorly vascularized areas. He concluded from these observations that glycogen "acts in the embryo as the precursor or deputy of hemoglobin until such time as the vascularity of the part is sufficiently advanced, and in other cases as the substitute for hemoglobin from first to last, i.e., in those tissues which are built up in whole or in part without the direct access of blood." Creighton's views have been sharply criticized by Sundberg (1924), largely because of his observations that the fetal parathyroid which he assumed to be richly vascularized was also rich in glycogen. Similarly, glycogen was absent in the renal tubules of both the mesonephros and metanephros, although the former, according to him, is richly vascularized and the latter poorly so. Since, however, it is now known that embryonic tissues are characterized by incomplete or deficient systems of respiratory enzymes, Sundberg's observations are not incompatible

with the view that glycogen deposition occurs in regions of limited aerobic metabolism. It is immaterial, according to this view, whether the limited aerobiosis is induced by a deficient blood supply, a limited complement of respiratory enzymes, or both. In place of Creighton's suggestion that glycogen acts as a substitute or an adjunct of hemoglobin, we would propose that it may provide, through anerobic glycolysis, a source of energy for oxidations in those tissues which are deficient in their mechanisms for aerobic metabolism.

The examples cited in the preceding paragraphs offer abundant instances in which glycogen deposition is associated with a deficient circulation or sluggish metabolism, or both. It should be pointed out, however, that glycogen also is deposited in at least two localities, namely, liver and muscle, in which the relationship is by no means so clear. In neither of these tissues is there a deficient blood supply nor a deficient complement of respiratory enzymes. On the other hand, an anerobic mechanism in muscle is known to exist, and the blood supply of the liver, although rich, is largely derived from the hepatic portal system in such a way that the hepatic cells are perfused with blood in which the oxygen saturation is lower than is the case in other tissues. Moreover, the situation is still further complicated in these organs by the fact that the glycogen content is easily altered by changes in the blood sugar concentration and by changes in endocrine balance. Consequently, it may be argued either that these organs contain special mechanisms superimposed upon the general one discussed above, or that they represent entirely separate special cases in which glycogen storage is determined largely by the relative rates of delivery and utilization of glucose.

The distribution of iron in the placenta

The significance of the iron content of the placenta centers around two principal topics. Since the iron of the fetus must be derived from the maternal blood, iron must traverse the placental barrier where it should be detectable. Moreover, since the syncytium is a region of active oxidative metabolism, iron-containing respiratory enzymes should presumably be present. If these considerations are valid, it follows that iron must be present in the placental barrier. Yet, previous attempts to demonstrate the element in the epithelial portions of primate placentae have led to only inconclusive evidence. Hofbauer (1905) has illustrated abundant quantities of iron in the fetal mesenchyme immediately underlying the syncytium, but does not show a Prussian blue reaction in the trophoblast itself, although he speaks somewhat ambiguously of having demonstrated it in the deepest portion of the syncytium. On the other hand, by using prolonged treatment with warm reagents, Fornera (1912) was able to demonstrate a Prussian blue reaction in the syncytium as well as in the stroma.

These considerations lead to the reflection that iron, if present in

the placental epithelium, must be so firmly bound into an organic molecule that drastic procedures are necessary for its unmasking. Of all the unmasking procedures for iron, incineration is the most complete, since the combustion of organic material leaves behind only the mineral residues as the non-volatile oxide. The iron oxide is easily detectable among the other mineral ashes because of its characteristic reddish or yellowish color. Consequently, microincineration provides the most complete method for the determination of iron, since it frees all iron from any organic combination (Policard, 1923; Scott, 1933b).

Iron, as revealed by the yellowish or reddish color of its oxide, was detected in the syncytial and cytotrophoblastic elements of the placenta, as well as within the fetal connective tissue of the villi. The syncytium exhibited a small quantity of iron dispersed as punctate aggregates of ash throughout the cytoplasm, and a denser concentration of ferrous material aggregated around all of the nuclei. On the other hand, the cytotrophoblastic cells were characterized by a heavy concentration of iron in the interstitial ground substance between them, whereas the cytoplasm and nuclear membranes contained very little.

It appears, as a likely hypothesis, that the perinuclear iron of the syncytium may represent that which has been organically bound into the respiratory enzymes. In support of this hypothesis are the following facts. The respiratory metabolism of the syncytium is greater than that of the cytotrophoblastic and decidual cells, and the amount of perinuclear iron is correspondingly greater. Contrariwise it seems unlikely *a priori* that the iron of passage should be intimately associated with the surfaces of the nuclei. Finally, the concentration of indophenol oxidase around the nuclei of the syncytium is remarkably similar to the distribution of iron in the same region. These considerations all lead toward the thought that the iron surrounding the nuclei is associated with the cytochrome system, while the iron scattered in the cytoplasm may represent iron of passage. It may be recalled, in this connection, that the Golgi apparatus of the syncytium, instead of being aggregated near the nuclear membranes as in the majority of cells which have been investigated, is disposed in the syncytial cytoplasm in the form of disconnected threads (Wislocki and Bennett, 1943). Thus its distribution bears no appreciably close relationship to the perinuclear deposits of iron.

SUMMARY AND CONCLUSIONS

The present study embodies the results obtained by the application of a number of histochemical reactions to human placental tissue. These reactions have centered around the localization of lipoids, glycogen and iron.

The lipoids of the fetal placenta occur almost entirely as droplets

in the syncytial trophoblast. These droplets apparently contain steroidal substances, since they give positive reactions characteristic of steroids. The reactions employed in this investigation consist of birefringence, lipoid-soluble autofluorescence, the Liebermann-Burchardt or sulfuric acid reaction, and the Schiff or plasmal reaction. The results obtained by these methods indicate that the syncytium is the site of localization of the placental steroid hormones.

The cytotrophoblastic cell columns, cell islands, and cytotrophoblastic shell contain histologically demonstrable glycogen especially during the early parts of pregnancy. Similarly, heavy deposits of glycogen occur in the maternal decidua in the first half of pregnancy. The glycogen-containing tissues in these localities are characterized by a relative or complete avascularity and by deficient mechanisms for aerobic oxidation. From consideration of glycogen distribution in various tissues, both fetal and adult, the concept is developed that glycogen deposition is generally associated with anerobic conditions, and that, through anerobic breakdown, it may provide a mechanism for the oxidative reactions of tissues in which the oxygen supply is deficient.

It was demonstrated by the Turnbull blue reaction, as well as by the study of microincinerated sections, that the syncytium of early placentae contains considerable quantities of iron, while in later stages of pregnancy the amount is greatly reduced. A dense concentration of iron exists in the intercellular matrix associated with the cytotrophoblastic cells in the various localities where they occur. These findings are briefly discussed in relation to the topic of iron transport by the placenta.

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THE COMPARATIVE BIOASSAY OF SEVERAL EXTRACTS OF THE ADRENAL CORTEX IN TESTS EMPLOYING FOUR SEPARATE PHYSIOLOGICAL RESPONSES[1]

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IN FEBRUARY 1942, our laboratory was invited to undertake for the Committee on Medical Research a quantitative biological comparison of certain extracts of the adrenal cortex. The objectives outlined for us by the Committee were: (a) the determination of the most reliable criteria of total adrenal cortical activity in adrenal cortical extracts; (b) the standardization of the assay procedure(s) in order to insure its (their) reproducibility in other laboratories; and (c) the evaluation of the potency of the several extracts in terms of suitable standards. Because of the divergent manifestations of adrenal cortical activity in the animal body it was necessary to select several bioassay procedures based upon different physiological responses for the comparisons. The methods finally chosen were: (a) the test of growth and survival in immature adrenalectomized rats as described by Grollman (1941); (b) the test of renal function in adrenalectomized dogs as proposed by Pfiffner, *et al.* (1934); (c) the test of sodium retention in normal dogs as devised by Hartman, *et al.* (1941); and (d) a test of glycogen deposition in fasted, adrenalectomized rats based upon principles recommended by Britton and Silvette (1932) and developed by Reinecke and Kendall (1942).[2]

Since the first three methods of assay were employed by us without significant departure from the original procedures, it is necessary in this paper to consider only the assay of the carbohydrate factors in detail. In the following treatment, a section is devoted to each of the methods of assay.

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[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and St. Louis University.

[2] At the time of the initiation of this work the glycogenic assay of Reinecke and Kendall (1942) was undergoing development. At the request of the Committee on Medical Research, a representative of our group was sent to the laboratory of Dr. Kendall to secure information about his method. We are appreciative of Dr. Kendall's kind cooperation.

THE GLYCOGENIC POTENCY OF ADRENAL CORTICAL EXTRACTS IN FASTED, ADRENALECTOMIZED RATS

After an extended consideration of the various manifestations of the influence of cortical extract upon carbohydrate metabolism, and a certain number of preliminary experiments, it was decided that a method based upon the glycogenic principle employed by Reinecke and Kendall (1942) gave the most promise of successful application. Our continued finding of aberrant results with their original procedure, however, led us to devise certain modifications.

It is well known that under the physiological conditions prevailing in the intact animal, liver glycogen does not completely vanish even after the most prolonged fasting. Since these fasting glycogen stores are abolished by adrenalectomy and restored by cortical extract but not by adrenaline, it has been assumed that the secretions of the adrenal cortex are responsible for the maintenance of minimal glycogen levels in the livers of normal, fasted animals. That the process sponsored by these hormones in the preservation of this liver glycogen is gluconeogenesis from protein seems to be indicated by the experiments of Evans (1935), Long, *et al.* (1940), Wells (1940), and Wells and Kendall (1940). That this process is advanced in livers liberally supplied with amino acid substrate is indicated by the experiments of Guest (1941) and Mirski, *et al.* (1938). These latter workers found that the livers of normal animals fed high protein diets contained significantly more glycogen after a 24-hour fast than did those of normal animals fed high carbohydrate diets. These effects were increased in rats prefed high protein diets and fasted at a pressure of one-half an atmosphere (Guest, 1941), a procedure which is thought by Evans (1935) and Lewis, *et al.* (1942) to stimulate an increase in the secretion of adrenal cortical hormones. These findings made it seem likely to us that injected adrenal cortical principles would stimulate greater glycogen depositions in the livers of adrenalectomized rats if a high protein dietary regime preceded the periods of fasting and injection. Experiments to test this hypothesis have confirmed it and have led to the regular feeding of a high protein diet in the post-operative four day period.

Under the high protein regime, it has been observed that a strictly linear relationship over a wide range of doses exists between the logarithm of the total dose of adrenal cortical extract injected into a fasted test rat over a period of six hours and the mean amount of glycogen found in its liver two hours after the last injection. Numerous control experiments have pointed to the necessity of choosing rats at an age (60–75 days) at which the adreno-hepatic relationships are stabilized since it appears that "chemical maturity" affects gluconeogenesis as well as susceptibility to choline lack (Griffith, 1941) and the distribution of bodily constituents (Chanutin, 1931). The rate of glycogen deposition in our procedure is such that maximum liver gly-

cogen levels are attained 2–4 hours after the last injection. It is of considerable importance to restrict the fluid intake during the assay period, and to administer the hormones parenterally in saline or 10 per cent alcohol since the use of oil as a vehicle results in the apparent recovery of only 50 per cent of the activity. Neither supplementary ascorbic acid nor more frequent administration of the hormones was found to improve the response. Careful attention to the maintenance of these conditions resulted in improvements in both the total response of the animal to a given dose of cortical extract and the linearity of curve in which the logarithm of the dose is plotted against the response.

EXPERIMENTAL

Animals. Male albino rats from the highly inbred colony of this laboratory were employed in these experiments. In the course of these studies they were selected in groups at two age levels, 30–31 days, and 70–75 days. Only those animals which weighed 60–90 grams in the first instance and 145–180 grams in the second were considered acceptable.

Diets. Several diets were employed in the course of this study. The so-called low protein rations were: (a) the growth assay ration of Grollman (1941); (b) Purina dog chow; and (c) G-2, an artificial ration employed by Mulford and Griffith (1942) in the study of choline deficiency. It contained: casein 18, dried brewer's yeast 6, sucrose 48.7, lard 19, Hawk Oser (1931) salt mixture 4, calcium carbonate 1, codliver oil 1, cereal cellulose 2, cystine 0.3, and choline chloride 2 mg. per gram of food. The protein content of these diets ranged from 21–23 per cent. The high protein diet containing approximately 58 per cent protein was designated OG-2. It was produced by increasing the casein content of G-2 to 55 per cent at the expense of carbohydrate. Gram for gram replacement of carbohydrate by protein lowered the carbohydrate content of OG-2 to 11.7 per cent. All of the diets were of sufficient biological value to support good growth in immature rats.

Final assay procedure. Male albino rats in groups of 20–30 animals ranging in age from 60–75 days and in weight from 145–185 grams were removed biweekly from their stock fare of Purina dog chow, anesthetized with ether, bilaterally adrenalectomized by the lumbar route according to Grollman (1941) and then placed in raised bottom cages and given Diet OG-2 and tap water containing 1 per cent sodium chloride until the morning of the fourth post-operative day. They were then fasted until the morning of the fifth post-operative day at which time their drinking water was removed and the cortical injections begun. Each rat was given a fixed dose of extract either in saline or 10 per cent alcohol in four equally divided doses at two hour intervals. It was the usual practice to assay two extracts per day, arranging the injections so that each extract was given to groups of 4–6 rats at three dose levels. Two hours after the last injection, the animals were narcotized in batches of four with 1.5 cc. of 1 per cent sodium amytal intraperitoneally, the abdomens opened, and approximately one-half of the left lateral lobe of each liver dropped into a tared, tapered 50 cc. centrifuge tube containing 2.0 cc. of cold 30 per cent KOH (Cori, 1932). The samples were quickly weighed on a damped, chainomatic balance and transferred to a boiling

water bath until dissolution of the liver tissue became complete. One hour sufficed to extirpate, weigh, and dissolve the livers from such a group. The precipitation and hydrolysis of the glycogen was then carried out according to the directions of Good, Kramer, and Somogyi (1933). Total reducing substances in the neutralized diluted filtrates were determined by the method of Shaffer and Somogyi (1933) using reagent 50 containing 1.0 gm. of KI per liter. All glycogen values have been expressed in terms of their glucose equivalents. Control experiments testing the recovery of glycogen added to alkaline digests of liver tissue, the rate of glycogenolysis in sectioned, anesthetized liver tissue *in situ*, and the distribution of glycogen in the various lobes of the same liver showed that the errors associated with these variables were much less than the mean biological error inherent in the assay response. Losses of weight up to 15 per cent of the initial body weight in the five day period following adrenalectomy would be sustained without interference with the liver response. Only those animals which were moribund after the twenty-four hour fast were discarded. Diarrhea was an inconstant finding in our rats, occurring, without apparent effect upon the liver glycogen deposition, in about 5 per cent of our animals. Mortality from adrenal insufficiency in the post-operative period ranged from 5–10 per cent.

Extracts. All of the samples received for assay from commercial and private laboratories were kept at 4°C. No deterioration in the biological potency of any of them was noticed over a period of eighteen months. These extracts are referred to by different Roman numerals in the text and charts.

Standard. It is suggested that corticosterone be adopted as the reference standard for the glycogenic assay. This C_{11}-hydroxy steroid is highly active in inducing glycogen deposition in fasted, adrenalectomized rats as well as being comparably active in other biological tests of cortical activity (Reichstein, *et al.* (1943). We have defined a unit of glycogenic activity as the potency of one microgram of corticosterone administered to a fasted, adrenalectomized rat in four divided doses at two hour intervals. Since this amount of corticosterone is actually below the threshold of response in our rats, various multiples of one microgram, equivalent, then, to as many units, were administered in order to get measurable depositions of glycogen. All expressions of glycogenic potency in our experiments have been made with reference to corticosterone. In a following section the potency of the cortical extracts tested by us is recorded in terms of units per cc. and per gram equivalent of fresh adrenal tissue.

RESULTS

I. *The effect of age and diet upon the degree of deposition of liver glycogen in the adrenalectomized rat, and the nature of the relationship between this deposition and the quantity of cortical extract injected.* Male albino rats were removed from the stock colony fare of Purina dog chow on either their thirtieth or their sixtieth to seventieth day of life, adrenalectomized, transferred to either a high or low protein[3] diet and 1 per cent saline drinking water for four days, fasted for 24 hours, and then injected with given amounts of Extracts II, III, and

[3] Low protein, as used in this report, refers to diets in which the protein content varied from 21–23 per cent.

IV. Two hours after the last injection they were narcotized and a portion of liver taken for glycogen analysis. The results of these experiments, together with the significances (Fisher, 1932) of the differences observed between rats prefed high- and low-protein diets are included in Table 1.

TABLE 1. THE EFFECT OF PREFEEDING HIGH AND LOW PROTEIN DIETS TO FASTED, ADRENALECTOMIZED RATS INJECTED WITH ADRENAL CORTICAL EXTRACTS

Age	Weight	Diet	Dietary protein content	Extract no.	Total dose	Rats no.	Mean glycogen deposition	Significance of differences
days	*gm.*		%		*cc.*		%	*t*†
30–31	60–90	Grollman	21	II	1.0	4	0.10 ± 0.01*	
					2.0	12	0.33 ± 0.07	
					4.0	12	0.64 ± 0.10	
					6.0	4	0.70 ± 0.09	
30–31	60–90	OG-2	58	II	1.0	8	0.27 ± 0.06	2.83
					2.0	8	0.66 ± 0.06	3.56
					4.0	7	1.05 ± 0.23	1.64
60–75	145–180	Grollman	21	II	1.0	4	0.18 ± 0.04	
					2.0	16	0.32 ± 0.08	
					4.0	7	1.05 ± 0.12	
60–75	145–180	OG-2	58	II	1.0	46	0.31 ± 0.02	2.89
					2.0	68	0.76 ± 0.03	4.80
					4.0	37	1.22 ± 0.06	1.27
60–75	145–180	G-2	21	III	1.0	9	0.15 ± 0.04	
					2.0	9	0.50 ± 0.10	
60–75	145–180	OG-2	58	III	1.0	20	0.42 ± 0.04	4.78
					2.0	28	0.88 ± 0.04	3.53
60–75	145–180	Purina dog chow	23	IV	1.0	9	0.60 ± 0.06	
					2.0	9	1.35 ± 0.13	
					4.0	8	1.84 ± 0.08	
60–75	145–180	OG-2	58	IV	1.0	22	0.83 ± 0.05	2.84
					2.0	13	1.53 ± 0.12	0.99
					4.0	6	2.14 ± 0.07	1.93

* The deviations are expressed in terms of the standard error of the mean;

$$\sigma = \sqrt{\frac{Ed^2}{n(n-1)}}$$

† t is the significance factor of Fisher (1932) calculated for small groups of animals according to Burn (1937). A factor greater than 2.0 is assumed to indicate a significant difference in the responsiveness of corresponding groups.

These results clearly show that high protein feeding during the four day post-operative interim significantly increases the deposition of liver glycogen in fasted, adrenalectomized rats injected with cortical hormones. As shown in Table 1, the increase in liver glycogen deposition found in rats prefed high protein diets is greatest at low dosages of cortical extract. It is also more consistently shown in the rats 60–70 days old. Under various conditions neither normal nor adrenalectomized 30-day rats store liver glycogen as well or as uniformly as

do the older animals. It was found, for example, in separate experiments employing nine animals each that the mean 24-hour fasting liver glycogen level in normal rats, 30–days of age which had been prefed diet OG-2, was 0.18 ± 0.07[4] per cent while in normal rats, 60 days of age under the same conditions of feeding and fasting, it was 0.51 ± 0.12 per cent. This age differential in glycogenic function persists in adrenalectomized rats given cortical extracts, as shown in Table 1. For these reasons rats varying from 60–75 days in age and from 145–185 grams in weight were considered to be the animals of choice; all routine comparisons of the glycogenic potency of extracts and compounds have been made in animals of this age and weight.

It must be pointed out, however, in connection with the high protein effect that the same margin of improvement in the response of rats prefed high protein diets to injected cortical extract is not found with all extracts. With certain preparations of higher potency the differences in deposition found after high- and low-protein feeding may hardly be considered significant at the higher dose levels. This is well illustrated by the results obtained with Extract IV in Table 1. These differences in improvement of response may possibly be attributable to qualitative differences in the composition of the cortical extract given.

By plotting the degree of deposition of liver glycogen in terms of its per cent of fresh liver weight against the logarithm of the total dose of cortical extract injected into the fasted, adrenalectomized 60-day rat prefed a high protein ration, a linear relationship was observed over a wide range of doses. Low protein feeding during the postoperative interim results, generally, in non-linearity and smaller deposition. These relationships are graphically pictured in Figure 1. No strikingly constant relationship between total liver glycogen and the dose of cortical extract injected in adrenalectomized rats of widely varying size and age such as that reported by Bergman and Klein (1943) was found.

II. *The effect of solvent and mode of administration upon the glycogenic response.* Four solvents and two modes of administration were tested in a series of experiments. The effects of both enteral and parenteral administration of several extracts and crystalline hormones of the cortex in saline, 10 per cent alcohol, propylene glycol, and benne oil upon the glycogenic response in adrenalectomized rats fed, fasted, and injected in accordance with the standard procedure were carefully observed. The results of these observations are tabulated in Table 2. The benne oil solutions of the ether-soluble material from Extract II were prepared in the following manner. The whole extract was concentrated to dryness either by lyophilization (Hays, 1942) at 0° or distillation at 40°C. The dry residue was then exhaustively ex-

[4] All deviations in this paper are expressed in terms of the standard error of the mean.

tracted with peroxide-free ethyl ether at its boiling point, the combined washings filtered through paper, evaporated to dryness on the water bath, and the total solids determined. The pale yellow oil was then redissolved in a minimum amount of ether, a predetermined amount of benne oil solvent added, and the whole evaporated to constant weight on the boiling water bath. The solvent was added in

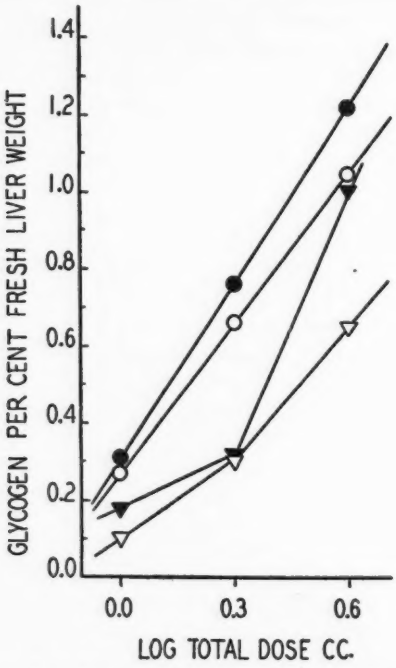

FIG. 1. The effect of age and diet upon the deposition of liver glycogen in fasted, adrenalectomized rats treated with cortical extract. ● Sixty day rats fed high protein diets; ○ 30 day rats fed high protein diets; ▼ 30 day rats fed low protein diets; ▽ 30 day rats fed low protein diets.

such proportions that the desired total dose was contained in 0.1–0.2 cc. which was then administered to fasted adrenalectomized rats in two doses at four hour intervals. The animals were sacrificed four hours after the last injection and the liver glycogen determined in the usual way.

It may be concluded from the data presented in Table 2 that there is no significant difference in the responses of fasted adrenalectomized rats to subcutaneous injections of cortical hormones in either saline or 10 per cent alcohol providing the alcoholic vehicle supplies less than 0.5 cc. of 95 per cent ethanol to the animal in the six hour period. Administration of aqueous or alcoholic extracts by stomach tube

resulted in the recovery of only 20 per cent of the glycogenic activity found after administration of the same extracts by the subcutaneous route. This drop in apparent activity was not nearly as marked when solutions of the pure steroids were used. Subcutaneous administration of benne oil solutions of the lipid-soluble substances from cortical Extract II gave recoveries ranging from 31–58 per cent of the "aque-

TABLE 2. THE EFFECT OF SOLVENT AND MODE OF ADMINISTRATION UPON THE APPARENT ACTIVITY OF ADRENAL CORTICAL HORMONES GIVEN TO FASTED, ADRENALECTOMIZED RATS

Route	Extract no.	Volume of extract	Solvent	Lipoidal solids	Rats no.	Mean glycogen deposition	Recovery of activity	Significance of differences
		cc.		*mg.*		%	%	††
Subcut.	I	1.0	0.9% saline		8	0.88 ± .08*	104	
		1.0	10% alcohol		8	0.87 ± .10	102	0.08
Subcut.	IV	1.0	0.9% saline		47	0.88 ± .05	100	
		1.0	20% propylene glycol		2	3.70 ± .20	810	13.76
		0.0	20% propylene glycol (1.0 cc.)		3	0.31 ± .04	—	
Subcut.	II	2.0	0.9% saline		22	0.77 ± .05	100	
		2.0	10% alcohol		11	0.81 ± .06	107	0.51
		4.0	0.9% saline		12	1.22 ± .05	100	
		4.0	10% alcohol		8	1.09 ± .10	85	1.16
Per os	II	4.0	10% alcohol		4	0.16 ± .09	20	10.30
Subcut.	II	1.0	benne oil	0.8	4	0.15 ± .04	40	3.58
		1.0	benne oil	0.9	4	0.14 ± .03	36	4.74
		1.0	benne oil	1.1†	3	0.12 ± .03	31	5.25
		2.0	benne oil	1.8	4	0.37 ± .10	55	3.75
		2.0	benne oil	2.2†	3	0.26 ± .03	47	11.80
		3.4	benne oil	3.1	4	0.75 ± .08	59	2.70
		7.0	benne oil	3.5	2	1.24 ± .07	58	1.72
		4.0	0.9% saline	—§	8	0.02 ± .01	0	
Per os		7.0	benne oil	3.5	4	0.40 ± .16	17	5.58
Subcut.	Compound A**		10% alcohol	0.40	4	0.46 ± .10	100	
	A		25% alcohol	0.44	4	0.40 ± .10	87	0.42
	E		20% propylene glycol	0.46	2	2.30 ± .21	600	7.47
Subcut.	B ± F		10% alcohol	0.34	7	0.95 ± .15	100	
	B ± F		benne oil	0.34	3	0.84 ± .20	82	0.44
Per os	B ± F		10% alcohol	0.34	3	0.70 ± .12	64	1.32
Per os	Glucose (200 mg.)		water‡	—	2	1.02 ± .25	100	
	Glucose (200 mg.)		water‡	—	4	0.35 ± .15	34	2.32

* The deviations are expressed in terms of the standard error of the mean.
† These fractions were injected in a single dose of 0.1–0.2 cc. of benne oil and the rats autopsied 6 hours after that injection.
‡ The glucose was administered in four equally divided doses at two hour intervals to normal rats which had been fasted 24 hours.
§ In this experiment the fat-free solids after extraction with ether were made up to their original volume with water.
** The letter designations of Kendall are used for the crystalline steroids.
†† Significance of the differences between glycogen depositions found with subcutaneous injections of cortical extract in saline and with test solvents as indicated.

ous" activity. When the crystalline steroids were administered in benne oil in subcutaneous injections, however, there was a quantitative recovery of the activity (Table 2). The administration of the lipid-soluble substances from cortical extract in benne oil *per os* resulted in the recovery of about 17 per cent of the "subcutaneous, aqueous" activity. This figure was raised to 64 per cent when crystalline steroids were used. The use of propylene glycol as a solvent for these hormones is to be discouraged because the solvent itself is glycogenic in fasted

adrenalectomized rats and gives augmented glycogen depositions when used as a solvent for cortical hormones. Injections of Extract IV and of crystalline 11-dehydro-17-hydroxycorticosterone in 20 per cent propylene glycol gave recoveries of 810 and 600 per cent of the predicted "aqueous" activity, respectively.

The water-soluble materials remaining after thorough ether extraction of desiccated cortical extract are entirely inactive as has been reported for the same after chloroform extraction by Kendall (1942).

In the assay of glycogenic factors by this procedure, the parenteral route is to be preferred. The poor recovery after enteral administration may be related to the intestinal changes after adrenalectomy which already have been described by Verzar, *et al.* (1936). Our own studies show that alimentary glucose is less glycogenic in the adrenalectomized rat than in the normal rat under fasting conditions (Table 2). The necessity for parenteral administration of cortical extracts and C_{11}-oxidized steroids in tests of growth in immature, adrenalectomized rats is apparently much less urgent (Kuizenga, *et al.*, 1940).

III. *The comparative activity of seven extracts of the adrenal cortex.* Five cortical extracts of beef and two of hog adrenals were tested for their glycogenic activity by our standard procedure. All injections were made subcutaneously into the nape with either saline or 10 per cent alcohol as the vehicle. Each extract was assayed at three and commonly four or five dose levels in order to define the position of its regression line with precision. Both the slope and the displacement of the individual regression lines from that of corticosterone were found to be of characteristic importance. The divergence in the slope of some of the regression lines from that of corticosterone made it necessary to select a level of glycogen deposition for the potency comparisons. A level of 1.00 per cent liver glycogen was arbitrarily chosen. This level was considered optimum for our colony of rats since it fell well beneath the ceiling of response (3.00 per cent) and at the same time was well above the fasting control base-line of $0.032 \pm .003$ per cent obtained in 56 rats. By application of the method of least squares (Dunn, 1929) to the glycogen deposition values obtained in individual rats injected with various doses of each extract, the formulae for the respective regression lines were calculated and appear in Table 3. Application of the same method to the glycogen deposition data obtained in 23 rats injected with doses of corticosterone varying from 0.4–1.2 mg. revealed the equation of the standard regression line to be:

$$y = 2.06 \pm 0.29 \text{ (logarithm dose mg.)} + 1.24 \pm 0.05$$

where y is the quantity of liver glycogen in terms of its per cent of fresh liver weight (Olson, *et al.*, 1944). By solving this deposition equation for 1.00 per cent liver glycogen, the mean dose required to

deposit this level of glycogen was found to be 0.766 ± 0.039 mg. Since the unit of glycogenic activity, as defined, is contained in one microgram of corticosterone, it follows that 766 ± 39 units are required to secure a level of 1.00 per cent liver glycogen in the test rat, and that

TABLE 3. GLYCOGEN DEPOSITIONS OBTAINED WITH SEVEN EXTRACTS OF THE ADRENAL CORTEX

Extract no.	Rats no.	Total dose	Mean glycogen deposition per cent	Regression line coefficients†	
		cc.		$b \pm \sigma_b$§	$a \pm \sigma_a$
I	18	1.00	0.82 ± .05*		
	24	2.00	1.45 ± .09		
	4	4.00	1.88 ± .15	1.92 ± .25	0.84 ± .05
II	46	1.00	0.31 ± .02	1.50 ± .10	0.31 ± .02
	68	2.00	0.76 ± .03		
	37	4.00	1.22 ± .06		
III	20	1.00	0.42 ± .04		
	28	2.00	0.88 ± .04		
	4	4.00	1.20 ± .10	1.41 ± .17	0.44 ± .03
IV	6	0.50	0.30 ± .06		
	22	1.00	0.83 ± .05		
	13	2.00	1.53 ± .12		
	6	4.00	2.14 ± .07	2.15 ± .15	0.88 ± .04
V	4	1.00	0.09 ± .02		
	10	2.00	0.39 ± .08		
	5	4.00	0.89 ± .13		
	2	8.00‡	1.25 ± .07	1.35 ± .20	0.03 ± .05
VI	9	0.25	0.33 ± .05		
	21	0.50	0.87 ± .07		
	37	1.00	1.57 ± .06		
	17	2.00	2.21 ± .10		
	6	4.00	2.93 ± .18	2.20 ± .08	1.57 ± .04
VII	4	1.00	0.09 ± .04		
	14	2.00	0.30 ± .05		
	7	4.00	0.30 ± .03	0.31 ± .19	0.16 ± .03

* The deviations are expressed in terms of the standard error of the mean.

† The regression line coefficients are for the equation from which glycogen in per cent fresh liver weight may be predicted from the logarithm of the dose (Bliss, 1943) $y = b$ (log dose mg.) $+ a$.

§ The standard errors of the regression line coefficients are calculated according to Dunn (1929).

‡ In this experiment the quantity of extract was lyophilized to dryness and made up to 4.00 cc. with water before injection.

precisely this many units will be contained in that amount of extract which will deposit 1.00 per cent liver glycogen in the test rat. These amounts, for all of the extracts tested were calculated from their respective regression line equations and are found in Table 4 together with their potencies in terms of glycogenic units per cc. and per gram equivalent of fresh adrenal tissue.

Since there is good evidence at present for strain differences in the reactivity of rats to injected cortical hormones, it may happen that all laboratories engaged in the glycogenic assay of cortical extracts will be unable to compare them at the same level of glycogen in the liver. The use of a reference standard and the calculation of regression line formulae for both standard and unknown in the manner outlined precludes any great discrepancy in the unitage reported for a given

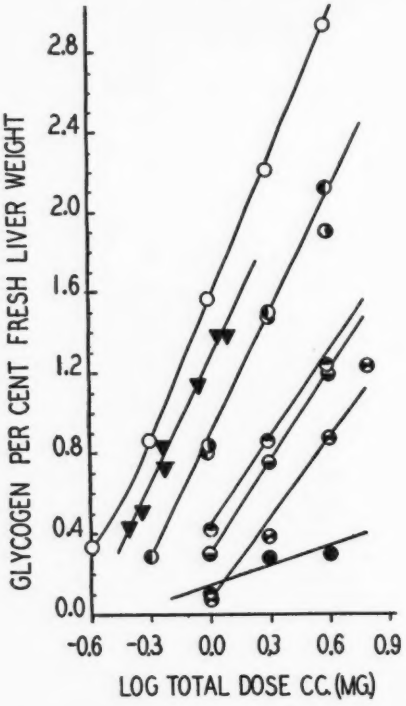

FIG. 2. Logarithm dose-response regression lines for seven extracts of the adrenal cortex and for corticosterone. ◑ Extract I; ◒ Extract II; ◓ Extract III; ◐ Extract IV; ⊖ Extract V; ○ Extract VI; ● Extract VII; ▼ Corticosterone. The abscissa units refer to the logarithm of the total dose of cortical extract in cc. in all cases but that of corticosterone in which the units refer to the logarithm of the total dose in milligrams.

extract regardless of the sensitivity of the particular strain. The relationships of the regression lines for seven extracts to each other and to the regression line of corticosterone are shown graphically in Figure 2.

There is a large variation in both the slope and position of the regression lines for the extracts tested (Tables 3 and 4). Extract VI from hog adrenals is by far the most potent per gram of fresh adrenal tissue. Extracts I and IV from beef adrenals are approximately one-

half as potent as Extract VI per gram of fresh adrenal tissue. All three of these extracts possess regression lines of high slope approximating that of the C_{11}-oxygen-bearing standard. The remaining Extracts II, III, and V from beef adrenals and Extract VII from hog adrenals are less potent and have regression lines of significantly smaller slopes. In spite of differences in the slope of their regression lines Extracts I and III are of comparable potency per gram equivalent of fresh adrenal tissue at a liver glycogen deposition level of 1.00 per cent.

TABLE 4. GLYCOGENIC POTENCY OF SEVEN EXTRACTS OF THE ADRENAL CORTEX IN TERMS OF CORTICOSTERONE

Extract no.	Rats no.	Amount required to deposit 1.00% liver glycogen in cc.	Potency per cc. (mg.)†	Potency per gram fresh adrenal tissue
			U.	U.
I**	46	1.21 ± 0.07	635 ± 55‡	8.5 ± .7
II*	151	2.86 ± 0.10	269 ± 21	6.7 ± .5
III*	52	2.49 ± 0.13	308 ± 23	7.7 ± .5
IV**	47	1.13 ± 0.05	682 ± 49	9.1 ± .7
V*	21	5.43 ± 0.47	142 ± 13	3.6 ± .3
VI**	90	0.55 ± 0.02	1400 ± 116	18.7 ± 1.6
VII*	25	59.4 ± 14.7§	7 ± 4	0.2 ± .1
Corticosterone	23	0.77 ± 0.04 mg.†	1000	

* The hormonal content of these extracts was equivalent to 40 grams of fresh adrenal tissue per cc.

** The hormonal content of these extracts was equivalent to 75 grams of fresh adrenal tissue per cc.

§ Because of the extremely low potency of Extract VII, comparisons were made at 0.40 per cent liver glycogen deposition instead of 1.00 per cent.

† (mg.) refers to the standard, corticosterone, only.

‡ The standard errors of the deposition equivalents and of the potency ratios are calculated according to the method of Bliss (1939).

These changes in slope and potency would seem to indicate not only a quantitative loss of important hormones in the preparation of some of these extracts, but qualitative alteration in their composition as well. It is surprising that two extracts of hog adrenal tissue (VI and VII) could differ so markedly both in potency and in the slope of their regression lines.

IV. *The precision of the assay as indicated by a statistical analysis of the results.* A statistical analysis of the results obtained in the glycogenic assays has been conducted according to the recommendations of Dunn (1929), Bliss, *et al.* (1939, 1943), and Burn (1937).

In assays of Extract II, involving four animals per assay in six separate months of the same year it was found that 2.0 cc. of extract gave liver glycogen depositions ranging from 0.21–1.17 per cent in individual rats, or a variation from the smallest response of 548 per cent. The mean deposition values for the groups of four in each month, however, varied only 44 per cent from the smallest mean (0.65–0.94 per cent liver glycogen) with an average deviation from the mean of the entire series (0.78 ± 0.05 per cent liver glycogen) of

only 8.3 per cent. Since none of these deviations gave a significance factor, t, greater than two, the errors were deemed insignificant. Additional assays of Extract II at a level of 2.0 cc. increasing the number to 68 did not give a mean deposition (0.76 ± 0.03 per cent liver glycogen) significantly different from that obtained in the 24 tests cited above.

The regression line constants for the extracts tested together with their standard errors were calculated by the formulae included in Dunn (1929) (Table 3).

In order to determine the absolute precision of an assay involving the comparison of corticosterone with a given extract, the standard error of the logarithm of the ratio of potencies, s_M, was determined according to the equation of Bliss (1939). For comparisons of seven extracts with corticosterone, s_M varied from ± 0.0299 to ± 0.0391 exclusive of the tests with Extract VII which showed a regression line of such small slope that the formula was inapplicable. Computation of the antilogarithms of s_M gave, respectively, errors ranging from 93.3–107.2 per cent and 91.4–109.4 per cent of the calculated potencies. Although the span of error is not identical on each side of the mean, the difference is small (1–3 units) and the largest deviation is recorded in Table 4.

It may be concluded from these findings that the nature of the response of fasted, adrenalectomized rats to cortical therapy under the conditions imposed by the assay is such that the administration of corticosterone and an unknown extract to groups of twenty animals each will give a potency ratio which will not err by more than ± 10 per cent. Since s_M is calculated for $t = 1$ sigma, it is required that extracts must differ in potency by at least 20 per cent before they may be considered significantly different (Fisher, 1932). In like manner, a difference equivalent to twice the weighted standard errors recorded in Table 3 for the regression line constants is required for a significant difference. It would be unwise to consider any slope different from the standard slope (2.06 ± 0.29) unless it differed from 2.06 by more than ± 0.50. It may be seen that since the deviation of the slopes of the regression lines for Extracts I, IV, and VI are less than 0.5 from 2.06 and those for Extracts II, III, V, and VII are greater than 0.5 from 2.06, the first group of extracts possess lines of standard slope while the second group have lines of substandard slope.

In the approximation of potency differences of given extracts it may be shown (Bliss and Marks, 1939) on the basis of our assay data that five rats in each of two groups injected with standard and unknown respectively will give a potency ratio which will not err by more than ± 20 per cent.

DISCUSSION

The finding of a linear logarithm dose-response relationship in the assay of the glycogenic function of cortical extract places this method

in a steadily growing group of bioassay procedures in which the response, whether it be in terms of body weight (Coward, *et al.*, 1930), gland weight (Bulbring and Burn, 1935), mean bone ash content (Hume, *et al.*, 1932), or fall in blood sugar (Bliss and Marks, 1939) is proportional to the logarithm of the dose of physiological stimulant.

The discovery of significant differences in both the slope and the position of the regression lines for different extracts from the slope and position of the regression line for the standard, corticosterone, has led us to consider both slope and potency constants as characteristic indices of each extract. The finding of these differences in the slopes of regression lines plotted for various adrenal cortical extracts is not without parallel in the fields of endocrinology and nutrition (Deansely, 1935; Marks, 1940; Shrimpton, 1940). These differences may be most reasonably accounted for by assuming qualitative differences in the action or composition of the stimulants.

Our finding of the glycogenic superiority of hog over beef cortical mixtures prepared by the same method has been foreshadowed by the findings of Vars, *et al.* (1934) with the dog assay of renal function and those of Kuizenga, *et al.* (1943) with rat survival and muscle work tests.

THE POTENCY OF ADRENAL CORTICAL EXTRACTS IN MAINTAINING NORMAL UREA CLEARANCE IN ADRENALECTOMIZED DOGS

The second method of assay adopted by us for the evaluation of the selected extracts was the renal function test of Pfiffner, Swingle, and Vars (1934). Since the effects of adrenal cortical extract upon renal function and carbohydrate metabolism seem to be the result of the stimulation of independent physiological mechanisms which are controlled by separate fractions of the extract, a careful comparison of assay results by the two methods was particularly desirable.

Methods. Adult male dogs were adrenalectomized in two stages, placed in individual metabolism cages, and maintained upon an exclusive diet of Purina dog chow (1.25 per cent NaCl) fed to them in the early part of the afternoon. The daily food intake of these animals was approximately 300 grams although borderline deficiency states induced in certain phases of the tests resulted in poorer appetites and lower food intakes. The animals were allowed to drink water *ad libitum* and exercise in a pen three times a day.

Urea nitrogen was photometrically determined (Hoffman, 1940) upon whole blood drawn from the saphenous vein in oxalated syringes at intervals of from 5–7 days.

At the beginning of each assay period the adrenalectomized dogs were receiving more than enough cortical extract or desoxycorticosterone acetate (DCA) to maintain them in a normal condition. The cortical substances were administered daily in two equally divided subcutaneous doses. The cortical extract was given in saline and the DCA in 30 per cent alcohol. As the assay progressed, the dosage of cortical substance was reduced stepwise,

in 5–7 day periods, until urea retention was observed. A rise in blood urea-N, of 100 per cent was considered the end-point..

The unit of cortical activity in the adrenalectomized dog has been defined by Pfiffner, *et al.* (1934) as the "minimum daily kilogram dose of cortical hormone necessary to maintain normal physiological conditions in the adrenalectomized dog for a period of 7–10 days; the two criteria being the maintenance of normal weight and blood level of urea nitrogen." Instead of employing the "Pfiffner unit," which is a function of individual dog sensitivity to cortical hormones, we thought it would be preferable to adopt a unit based upon a reference standard. Since Reichstein (1938), Thorn, *et al.* (1939), Cleghorn, *et al.* (1941), and Remington, *et al.* (1941) have found

TABLE 5. BLOOD UREA-NITROGEN VALUES OBTAINED IN ADRENALECTOMIZED DOGS MAINTAINED WITH DESOXYCORTICOSTERONE ACETATE

Dog no.	Blood urea nitrogen in mg. %			
	1.5†	1.0†	0.7†	0.5†
A. min.	8.0	18.2	14.5	28.5
max.	15.5	20.2	26.2	32.9
mean	11.4	19.2	22.3	30.7
B. min.	11.5	10.3	21.0	22.7
max.	18.5	13.9	27.9	30.6
mean	15.1	11.9	25.7	26.6
C. min.	10.0	10.3	19.0	
max.	17.2	19.2	29.0	
mean	14.3	15.0	26.9	31.6*

† Dose of DCA per dog per day in mg.
* Each dog was bled 3–5 times on each dose level with the exception of Dog C on 0.5 mg. of DCA per day.

desoxycorticosterone acetate capable of supporting life in adrenalectomized dogs, and since our experiments fully corroborate theirs, we suggest that DCA be adopted as the reference standard for this assay. We have defined the unit as the anti-urea retention activity of one microgram of DCA in the bilaterally adrenalectomized dog. The minimum amount of DCA which will maintain the blood urea nitrogen with the physiological span of 14–18 mg. per cent in a given dog is the protective dose of standard for that dog; the minimum amount of extract which will maintain blood urea nitrogen levels within the same span is the protective dose of extract for that dog. The potency of the extract is then obtained by substituting the following formula:

$$\frac{\text{(protective dose of DCA in mg.} \times 1000)}{\text{(protective dose of extract in cc.)}} = \text{DCA units per cc.}$$

Since a prophylactic lag is sometimes noticed when dogs are transferred from DCA to cortical extract it is suggested that the first period in the assay of extract be eleven days instead of seven. The augmentatory effect of the previous treatment with DCA seems to be neutralized in four days.

Results. The results of preliminary experiments with desoxycorticosterone acetate in three adrenalectomized dogs are shown in Table

5. The mean values for each dog were obtained by averaging several determinations made during its maintenance on a given dose of DCA. The minimum and maximum blood urea nitrogen values obtained in these serial analyses are also included for each dog on each dose. It may be seen that the protective dose of DCA for Dog A is less than 1.5 mg. and greater than 1.0 mg.; for Dogs B and C, it is approximately 1.0 mg. The protective dose of standard was determined for all of the animals used in these assays. The potency of the various extracts in controlling renal function as indicated by this method are listed in terms of DCA units per gram of fresh adrenal tissue in Table 6. The feature of these results is their average uniformity.

TABLE 6. THE RENAL FUNCTION POTENCY OF SEVEN EXTRACTS OF THE ADRENAL CORTEX IN DCA UNITS

Extract no.	DCA units per gram equivalent of fresh adrenal tissue				
	Dog A	Dog B	Dog C	Dog D	Dog E
I	5.5±1*	5±1			
II	4±1	4±1	4±1	4±1	3±1
III	4±1	4±1	4±1	3±0.5	4±1
IV	4±1	4±1			
V	7±2	7±2		3±0.5	4±1
VI	6±1	4±1			
VII	5±1				

* The standard error of the mean calculated for small groups according to Burn (1937) on the basis of at least three responses per dog per extract.

DISCUSSION

The results of our assays by this method are in good agreement with those of Thorn, *et al.* (1939), Cleghorn, *et al.* (1941) and others using relatively low sodium chloride containing diets. The lower maintenance doses which have been reported by Pfiffner, *et al.* (1934), Cleghorn, *et al.* (1941) and Remington, *et al.* (1941) for both desoxycorticosterone acetate and extract have been found in dogs fed higher amounts of NaCl. The effects of differences both in animal sensitivity and dietary salt upon the assay results tend to be minimized by the use of a reference standard. In spite of carefully controlled standardizations, however, the variation in the assay results obtained for a single extract tested in several dogs may be excessive (Table 6). It would appear that a larger number of dogs is required for assays of statistical significance.

From the semi-quantitative evidence presented, it would appear that all of the extracts submitted to us under the auspices of the Committee are of comparable replacement value per gram equivalent of fresh adrenal tissue in the bilaterally adrenalectomized dog.

It may be concluded that in spite of the difficulties which have been enumerated, this assay procedure for the measurement of the

factors of the cortex which influence renal function serves to reveal a type of adrenal cortical activity. The adrenalectomized dog is particularly sensitive to compounds related to desoxycorticosterone.

THE POTENCY OF ADRENAL CORTICAL EXTRACTS IN CONTROLLING THE EXCRETION OF SODIUM IN NORMAL DOGS

The method of Hartman, Lewis, and Thatcher (1940) in which the effect of cortical extract upon the excretion of sodium in dogs is measured, was selected for the evaluation of those factors of the cortex which affect inorganic metabolism.

Methods. Both male and female dogs ranging in weight from 8–15 kilograms were used. After preliminary control experiments testing their degree of constancy and sensitivity in the assay had been conducted, four males and three females were selected from a group of ten males and ten females. The males were segregated from the females to avoid excitement. The females were fed the beef heart mixture of Hartman, *et al.* (1941) while the males were maintained upon Purina dog chow; both consumed their daily food in a period of fifteen minutes. The bioassays were conducted according to the recommendations of the authors (Hartman, 1941) in both hot and cold weather and the results compared. Desoxycorticosterone acetate was employed as the standard in the place of desoxycorticosterone and administered to the dogs in a solution containing 10 per cent alcohol, 10 per cent propylene glycol, and 80 per cent water. The solvent was found to exert no influence upon sodium excretion. Urinary sodium was determined by the method of Butler and Tuthill (1931).

Results. In control experiments on normal dogs over a period of eight months the degree of variation in sodium excretion under physiological conditions was determined. Typical of the results obtained are the following two examples: Dog LBM showed a mean sodium excretion of 14.3 ± 1.9 mE of Na in a series of twelve tests on different days in the summer, and an excretion of 17.6 ± 2.3 mE of Na in a similar series in the winter. Dog BM excreted 23.7 ± 1.9 mE of Na in the summer series and 30.0 ± 1.1 mE of Na in the winter series. Both dogs were males. Although the mean values are significantly different in the second case, the degree of variation as revealed by their standard errors was not appreciably altered by the hot weather in either case. Indifference to the season was also seen in experiments in which the degree of sodium retention induced by standard amounts of DCA was measured. Retention of sodium by Dog LBM per 0.7 mg. of DCA in the summer was 40.1 ± 4.1 per cent in four tests; retention by the same dog per 0.7 mg. of DCA in the winter was 45.7 ± 2.6 per cent in five tests. In similar tests of Dog BM the retention of sodium in the summer was 48.4 ± 3.1 per cent while retention of sodium in the winter was 51.7 ± 3.6 per cent. The mean retention of sodium per 0.7 mg. of DCA in sixteen assays in both the winter and the summer on six dogs was 51.0 ± 1.4 per cent. The extracts were run in series

with DCA on each dog after a "rest" of two days. Only those runs in which 0.7 mg. of DCA induced a percentile retention of sodium within the "sensitive range" of 35–65 per cent were considered satisfactory for the calculation of the potency of an unknown extract. It was occasionally found that the same dose of DCA on successive runs would cause percentile retentions of sodium which fell both inside and outside of the sensitive range.

The results of the assays of four commercial extracts are listed in Table 7. The potency of these extracts is expressed in Hartman DCA units per cc. of extract. The variation is presented in terms of the standard error of the mean calculated by orthodox methods (Dunn, 1929). Even though the experiments here reported are too few to be properly subject to statistical analysis (See t values, Table 7), it is

TABLE 7. THE SODIUM-RETAINING POTENCY OF FOUR EXTRACTS OF THE ADRENAL CORTEX IN DCA UNITS

Extract no.	Assays no.	Dogs no.	Potency per cc.§	Potency per gram of fresh adrenal tissue	Significance of differences
			U.	U.	t†
I	12	6	1.53 ± .11*	0.021 ± .002	0.36
II	9	4	1.58 ± .16	0.040 ± .004	3.28
III	3	2	1.42 ± .31	0.036 ± .008	1.80
IV	2	2	1.42 ± .33	0.019 ± .005	0.00

* The deviations are expressed in terms of the standard error of the mean.
† The t factors are calculated with respect to the least potent extract, IV.
§ The unitage per cc. is calculated by the formula of Hartman, *et al.* (1941):

$$\text{U./cc.} = \frac{\%\text{ retention by unknown}}{\%\text{ retention by 0.7 mg. DCA}} \cdot \frac{10}{\text{volume of extract injected}}.$$

probable that Extracts II and III are more potent in sodium-retaining substances per gram equivalent of fresh adrenal tissue than are Extracts I and IV. Our data indicate that only slight changes in sodium-retaining potency are effected by different methods of preparation of extracts in four cases.

DISCUSSION

The control observations on sodium excretion in selected assay dogs during the summer months showed that although a slightly diminished excretion occurred in the summer, the variability in the results was approximately the same. Males and females showed no significant differences. The sodium retention responses to DCA were constant to the degree claimed by Hartman, *et al.* (1941). The retention responses to extract, however, although reasonably constant in a particular dog were highly variable in different dogs. Apparent differences in different dogs persisted in spite of standardizations with DCA in each case. The statistical significance of the differences is questionable in view of the small number of tests. On the basis of

the evidence presented it would appear that the Na-retaining factor of Hartman (1940) is present in four adrenal cortical extracts in approximately the same amounts.

In view of the separation of the Na-retaining factor from the "essential" cortin fraction by Hartman and Spoor (1940), it is doubtful that one is able to predict the replacement value of a given adrenal cortical extract on the basis of its sodium retaining power in the normal dog.

THE POTENCY OF ADRENAL CORTICAL EXTRACTS IN STIMULATING GROWTH OF IMMATURE, ADRENALECTOMIZED RATS

In order to test the effect of the selected adrenal cortical extracts upon the growth and survival of immature, adrenalectomized rats, the procedure of Grollman (1941) was followed without modification.

Methods. Young male rats were weaned at the age of 20 days and placed upon Grollman's diet for 10 days. At the end of this time the animals weighing less than 50 gm. were rejected and the others bilaterally adrenalectomized according to the technique of Grollman (1941). A few hours after the operation the animals were weighed and then injected subcutaneously with graded quantities of adrenal cortical extract or desoxycorticosterone acetate. The extracts were dissolved in either saline or 10 per cent alcohol while the DCA vehicle contained 10 per cent alcohol, 20 per cent propylene glycol, and 80 per cent water. On each of the six following days the injections were repeated and on the seventh day the final weight recorded. The mean weight gain was calculated from the initial and final weights of the individuals in the assay group and plotted against the logarithm of the daily dose of extract given. All animals surviving more than two weeks after the cessation of injections were not considered in the calculations of potency. Adrenal cortical remnants and accessory tissue were found in such surviving rats. In several experiments other diets than Grollman's were tested but none permitted the development of the deficiency state after withdrawal of extract as quickly as did Grollman's diet.

Calculations of potency were made in terms of desoxycorticosterone acetate which was arbitrarily assigned a standard potency of 1000 growth units per milligram.

Results. The growth responses of young adrenalectomized rats given adrenal cortical hormones parenterally in this procedure were extremely variable. Table 8 contains a summary of growth tests conducted on three hundred and seventy 30-day adrenalectomized rats over a period of eight months. There was no noticeable seasonal change in the sensitivity or the variability of our animals in these assays. The mean growth responses were plotted against the logarithms of the daily doses of extract or DCA given, and are shown in Figure 3. The regression lines were approximately by standard methods (Dunn, 1929) and drawn. The equation found for that of the standard DCA was:

$$y = 30.8 \pm 4.4 \text{ (log dose } \mu\text{g.)} - 47 \pm 1$$

where y is the mean growth in grams of body weight per seven days.

Because of the marked differences in the slopes of the regression lines for the standard and for the unknown extracts, it was arbitrarily decided to make potency comparisons at a growth level of 10 grams in seven days. By substituting 10 for y in the above equation it may be shown that 70 ± 8 micrograms of DCA were required to induce the

TABLE 8. GROWTH STIMULATION OBTAINED WITH FIVE EXTRACTS OF THE ADRENAL CORTEX AND WITH DESOXYCORTICOSTERONE ACETATE IN IMMATURE, ADRENALECTOMIZED RATS

Extract no.	Rats no.	Daily dose	Mean growth
		cc.	*gm.*
I	5	0.1	3.9±2.1‡
	14	0.2	1.5±1.4
	16	0.3	5.1±1.7
	12	0.4	4.5±1.4
	8	0.5	8.1±2.4
II	14	0.1	3.1±1.3
	7	0.15	4.1±2.0
	23	0.2	1.3±1.1
	13	0.3	8.2±1.7
	20	0.4	6.9±0.9
	56	0.5	5.7±0.7
	14	0.6	12.2±0.9
	39	1.0	7.8±0.7
III	15	0.4	3.8±1.8
	17	0.5	5.1±1.1
	15	0.6	3.9±1.0
	16	1.0	6.7±0.9
IV	24	0.3	1.8±1.1
	27	0.7	2.8±1.0
V	7	0.5	2.7±2.0
	8	1.0	4.4±1.4
DCA	6	0.04†	All died
	6	0.06	3.8±2.6*
	8	0.10	17.4±2.3
	9	0.15	21.6±1.2
	6	0.25	24.8±1.8

† The dosage of desoxycorticosterone acetate is expressed in terms of milligrams per day.

* One rat died.

‡ The deviation is the standard error of the mean calculated according to Burn (1937).

standard weight gain in our rats. Since DCA has been assigned a potency of 1000 growth units per milligram, it follows that the minimum amount of extract required to induce a weight gain of 10 grams in 7 days in our 30-day adrenalectomized rats would contain 70 ± 8 growth units. Calculations on this basis have been made for each extract and the potency values per cc. of extract and per gram equivalent of fresh adrenal tissue recorded in Table 9.

These results indicate that the selected extracts occupy the following sequence in order of potency per gram equivalent of fresh ad-

renal tissue: Extracts II, I, III, V, and IV. Because of the large variability in the responses of the animals, however, these mean potency values are subject to some qualification. It may be said that at least

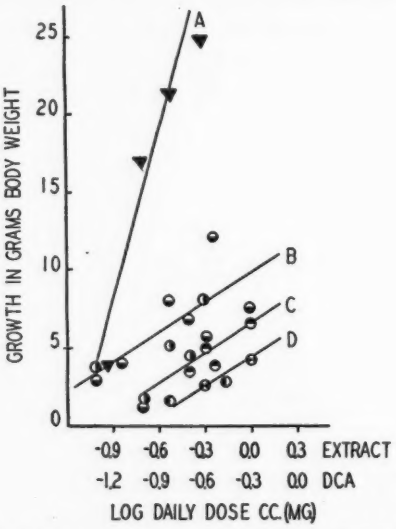

FIG. 3. Logarithm dose-response regression lines for five extracts of the adrenal cortex and for desoxycorticosterone in measurements of growth in immature, adrenalectomized rats. ◑ Extract I; ◒ Extract II; ◓ Extract III; ◐ Extract IV; ⊖ Extract V; ▼ Desoycorticosterone acetate; A is the regression line for desoxycorticosterone acetate. B the most representative line for Extracts I and II, C the same for Extract III, and D the same for Extracts IV and V. The abscissa units refer to the logarithm of the daily dose in cc. for the extracts and to the logarithm of the daily dose in mg. for desoxycorticosterone acetate.

a one-fold difference in potency exists between Extracts II and V, between Extracts II and IV, and between Extracts I and IV. Extracts I and II are only questionably different ($t = 1.5$) on the basis of equivalent adrenal tissue weights, while Extracts III, IV, and V are indistinguishable.

TABLE 9. THE POTENCY OF FIVE EXTRACTS OF THE ADRENAL CORTEX IN STIMULATING GROWTH IN IMMATURE, ADRENALECTOMIZED RATS

Extract no.	Rats no.	Potency per cc.	Potency per gram of fresh adrenal tissue	Significance of differences
		U.	U.	t†
I	55	58 ± 22*	0.77 ± 0.30	2.07
II	186	66 ± 20	1.70 ± 0.50	3.12
III	63	20 ± 8	0.50 ± 0.20	1.75
IV	51	10 ± 5	0.13 ± 0.07	0.00
V	15	12 ± 8	0.30 ± 0.20	0.80

* The standard error of the potency calculated by the method of Burn (1937).
† t of Fisher (1932) is calculated with respect to the least potent extract IV.

DISCUSSION

The large variation in the growth responses of similarly conditioned animals to the same dose of adrenal cortical extract or DCA significantly reduces the accuracy of this method. Although correlation coefficients in the neighborhood of 0.7 were obtained for the dose-response relationship when the mean values of Table 8 were used, analyses of individual growth responses showed values nearer 0.2 except in the case of DCA in which the value was 0.8. The scatter of responses around the calculated regression lines in Figure 3 and the magnitude of the standard errors recorded in Table 8 are further statistical evidence for this large variability.

In addition it must be pointed out that DCA has certain limitations as a reference standard for this method. For appropriate comparisons of the potency of an unknown with the standard, the slopes of the respective regression lines should be approximately the same (Bliss and Cattell, 1943). This is not so for DCA and the extracts in this procedure (see Figure 3). It was also found, as would be expected from the position of the regression line, that amounts of DCA which would give growth responses comparable to those obtained with extract were barely sufficient to keep the assay animals alive.

Kuizenga, *et al.* (1940) found that approximately 29 μg. of DCA per day induced weight gains of 1 gm. per day over twenty day periods and insured the survival of 80 per cent of their immature, adrenalectomized rats. Grollman (1939) on the other hand, has reported that while 200 μg. of DCA per day would give weight gains of 2 gm. per day and permit the survival of approximately 80 per cent of his young adrenalectomized rats, 1 mg. per day was required to permit complete survival and normal growth. Our data indicate that 67 ± 8 μg. of DCA daily will support a growth rate of 1 gm. per day and protect 80 per cent of our immature, adrenalectomized rats, but that 100 μg. daily are required to permit normal growth and complete survival.

One extract (IV), found to be very active in the glycogenic assay and of comparable activity to other extracts in tests of renal function was practically inactive in stimulating growth in young, adrenalectomized rats. The relative inadequacy of the C_{11}-substituted glycogenic steroids, or extracts containing them in large preponderance, in supporting growth in young adrenalectomized rats has already been reported (Grollman, 1939; Wells and Kendall, 1940).

COMPARISONS OF THE ADRENAL CORTICAL POTENCY OF SEVEN EXTRACTS DETERMINED BY FOUR METHODS

A summary of the results obtained with the four methods of assay expressed in terms of their respective units per gram equivalent of fresh adrenal tissue is given in Table 10. It may be seen that in spite

of the large variation in the glycogenic potency of the seven extracts, ranging from 18.7 ± 1.6 to 0.2 ± 0.1 units per gram equivalent of tissue, the potency in units of renal function is practically constant. Extracts I and IV, the most potent glycogenic extracts of beef glands, are relatively poor in growth and sodium-retaining potency. Extracts II and III which are slightly poorer in glycogenic potency and regression line slope show better sodium-retaining power. Extract II is the most potent in stimulating growth in immature, adrenalectomized rats. It is unfortunate that circumstances did not permit the assay of Extracts VI and VII in all respects.

In general, the extracts higher in glycogenic potency, i.e. those extracts containing a preponderance of C_{11}-oxygen-bearing steroids, are lower in growth and sodium-retaining factors. Extracts lower in glycogenic potency are generally higher in growth and sodium-retaining potency. This reciprocal relationship between glycogenic potency and the growth and sodium-retaining potency of these extracts parallels a similar reciprocal relationship in the physiological properties of desoxycorticosterone and the 17-hydroxycorticosterones (Thorn, *et al.*, 1941). Our results with the extracts are anomalous insofar as the renal function potency did not more closely parallel the growth and sodium-retaining potencies.

In spite of the variation in the factors affecting renal function, growth, and sodium retention recorded in Table 10, it may be seen that these factors are retained by all methods of preparation to a much more uniform extent than are the factors concerned with carbohydrate metabolism. It would appear to us, therefore, that the biological examination of all extracts for their content of glycogenic factors, which appear to be largely, if not solely the C_{11}-oxygen-substituted steroids (Olson, *et al.*, 1944) is important.

These studies, as well as others (Swingle and Remington, 1944) seem to indicate that there are two major "types" of activity in adrenal cortical extracts. The influence of cortical extract upon renal function and inorganic metabolism appears to be due to cortical steroids which are completely reduced at C_{11}, i.e., the desoxycorticosterones. The influence of cortical extract upon gluconeogenesis and other organic reactions, on the other hand, appears to be due to steroids which are partially oxidized at C_{11}, i.e., the corticosterones. Since these activities of cortical extracts are more or less independent, it follows that at least two methods of bioassay are required to characterize properly their physiological potency. Our results indicate that the test of glycogen deposition in fasted, adrenalectomized rats is satisfactory for the detection of the activity of the corticosterones. They indicate, further, that the tests of renal function in the adrenalectomized dog as indicated by an improvement in urea clearance, and in the normal dog as indicated by a retention of sodium, though less specific (Allers and Kendall, 1937; Thorn and Harrop, 1937), are

suitable for the determination of the activity of the desoxycorticosterones. We feel that the former method is to be preferred because of its use of an adrenalectomized animal. Our objections to the test of growth and survival in young adrenalectomized rats are based chiefly upon the large variability in the growth responses of these animals to injected cortical extracts. In addition to this growth *per se*

TABLE 10. THE COMPARATIVE ACTIVITY OF SEVEN ADRENAL CORTICAL EXTRACTS IN TERMS OF TWO STANDARDS IN FOUR METHODS OF ASSAY

Standard	Corticosterone	DCA	DCA	DCA
Extract no.	Glycogenic potency per gram†	Renal function potency per gram†	Potency in stimulating growth per gram†	Sodium retaining potency per gram†
	U.×10	U.	U.×10	U.×1000
VI**	187±16‡	5±2		
IV**	91±7	5±2	2±1	19±5
I**	85±7	5±2	8±3	21±2
III*	77±5	4±1	5±3	36±8
II*	67±5	4±1	17±5	40±4
V*	36±3	5±2	3±2	
VII*	2±1	5±1		

* The hormonal content of these extracts was equivalent to 40 grams of fresh adrenal tissue per cc.

** The hormonal content of these extracts was equivalent to 75 grams of fresh adrenal tissue per cc.

† Per gram equivalent of fresh adrenal tissue.

‡ The deviation is expressed in terms of the standard error.

is a highly cumulative assay end-point upon which desoxycorticosterone and the corticosterones may exert opposite and neutralizing effects (Wells and Kendall, 1940).

It is highly questionable, in view of this division in the effects of adrenal cortical hormones in the animal body, that a single criterion of total adrenal cortical activity will be found.

SUMMARY

Seven adrenal cortical extracts have been examined with four methods of bioassay, each dependent upon a different physiological response.

A modified Kendall procedure employing fasted, adrenalectomized rats which had been prefed a high protein ration was developed for the assay of those factors (C_{11}-oxygen-substituted steroids) of the adrenal cortex which influence carbohydrate metabolism. A wide variation in both the slope and the position of the regression lines relating the logarithm of the dose of cortical extract to the degree of glycogen deposition was observed in tests on seven extracts.

The Pfiffner, *et al.* (1934) test of renal function, the Hartman (1941) test of sodium retention, and the Grollman (1941) test of growth and survival were also employed in the assay of these extracts.

Corticosterone was used as the reference standard in the method for the assay of glycogenic factors and desoxycorticosterone acetate was used as the reference standard in the other three procedures.

One extract of hog adrenals was found to be twice as active in glycogenic potency per gram equivalent of fresh adrenal tissue as the most potent beef extract; another extract of hog adrenals was found to be virtually inactive in glycogenic potency.

Parallelism between the glycogenic activity and cortical activity determined by other methods was not observed in comparisons made on seven extracts.

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THE GROWTH OF THE ANTERIOR LOBE OF THE PITUITARY AND THE TESTES IN THE COCKEREL[1]

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THE GROWTH of the pituitary gland in normal animals during the period from birth or hatching to maturity has been given very little attention by endocrinologists. This situation is surprising in view of the excellent analysis of pituitary development and function in the foetal pig by Smith and Dortzbach (1929), and Nelson (1933), the detailed studies of the physiological activity of extracts and implants of the anterior pituitary, and the cytological studies of cell types which have been correlated with varied physiological conditions. The significant problems involving the amount and initiation of gonadotropic hormone secretion by the anterior lobe and the production of sex hormones by the gonads with their reciprocal action on the pituitary are yet to be investigated fully during this early period of hypophyseal development. The best available growth data for mammals are presented by Hatai (1914), King and Donaldson (1929) for the rat, Rasmussen (1938) for man, Stockard (1941) for the dog, and Bates, Riddle and Lahr for cattle (1935). Similar investigations by Latimer (1924), Landauer and Aberle (1935) for the White Leghorn, Juhn and Mitchell (1929) for the Brown Leghorn and Leonard and Righter (1936) for the Bantam are the most extensive for birds. Other classes of vertebrates are not sufficiently represented to warrant discussion.

Because of this gap in the history of the pituitary, it seemed appropriate to study one animal in detail in order to establish the normal developmental picture. The White Leghorn cockerel during the first 90 days post-hatching was chosen for this initial study of the growth of the pituitary gland. The time from hatching to 90 days of age is probably the most critical physiological interval in the White Leghorn: the secretion of the anterior pituitary gland and release of androgen by the gonads may begin as early as the 15th day, Payne (1942), Breneman (1941); as much as one-third of total body growth may occur; combs and gonads undergo relatively their greatest devel-

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opment; and spermatogenesis is initiated. Recent cytological studies of the Leghorn pituitary by Payne (1942) have established a clear picture of the cell history during most of this period. Riley and Fraaps (1942) have demonstrated that it is feasible to assay the gonadotropic potency of individual fowl pituitaries and it should be possible, therefore, to correlate growth, cell types, and physiological activity in these animals.

The inclusion of data for testicular weights in this account is important because these reflect the sex stimulating activity of the hypophysis and in turn the gonad secretions modify pituitary function. The present information available for birds is conflicting because Riddle (1931) has stated that the testes have a very slow growth in pigeons in the interval up to 1.7 months post hatching, although body growth is extensive; and that a longer period of slow growth, 2.5 months, is present in ring doves. Riddle and Schooley (1935) interpreted these facts as evidence of a lack of follicle-stimulating hormone in the pituitary during this period. These data would not be comparable, however, to the preliminary observations on testicular development in White Leghorns previously cited, nor to the observations for mammals in which gonadotropins were demonstrated in the pituitary of rats as early as 5 days after birth (Smith and Engle 1937).

MATERIAL AND METHODS

Only White Leghorn chicks purchased from one hatchery were used for this study. The chicks were received within twelve hours after hatching and were placed in a battery brooder immediately. The animals were fed a good stock ration to which was added 4% tankage in order to bring the ration up to pre-war standard. Although body weights were remarkably uniform, it was occasionally necessary to eliminate obvious "runts" but no other selection was practiced.

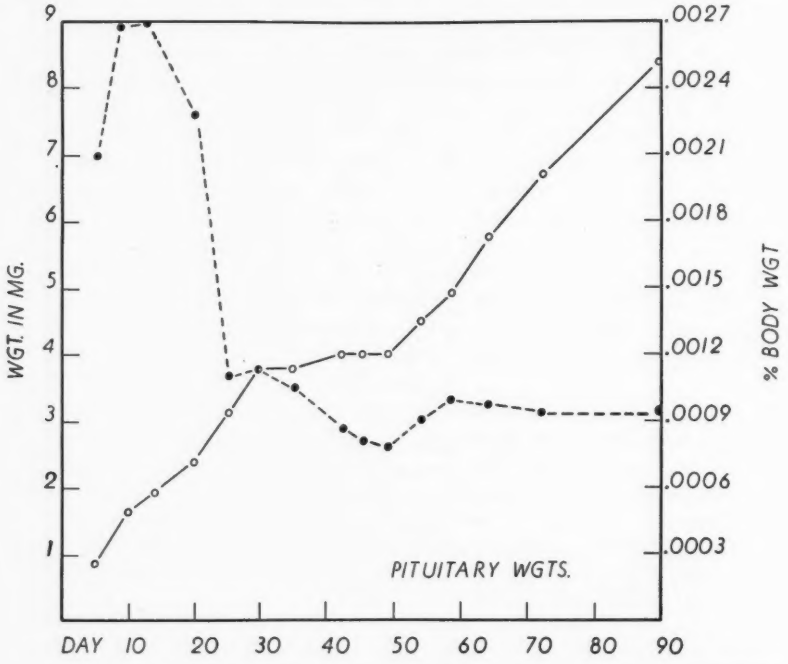
It was planned to take pituitary samples every five days, but since the glands were also used for injection, assay animals were not always available at the proper time; however the five-day intervals were used whenever possible. The glands were removed by severing the head and removing the lower jaw, following which a transverse cut was made with a scalpel across the roof of the mouth just posterior to the rostrum and the wedge of bone thus freed was pried anteriorly exposing the pituitary. The capsule, which was usually intact, was dissected free with the aid of jeweler's forceps, and the anterior lobe separated from the posterior. Individual glands were not weighed but were placed in a moist chamber and usually weighed in lots of ten. The time required to kill ten birds, remove and weigh a group of glands was about five minutes. The obvious disadvantage of the method employed is that it does not allow the calculation of standard errors, but a more accurate mean weight probably can be determined by this method. This would be particularly true of young birds in which the anterior lobe is very small. All weighing was done on a chainomatic balance.

Known amounts of pituitary tissue were placed in acetone which was changed three times. Upon removal from the acetone the glands were ex-

posed to the air at room temperature until thoroughly dried and then weighed. This figure was then used for the calculation of the "acetone dried weight."

RESULTS AND DISCUSSION

Growth of the anterior lobe. The data for the growth of the anterior lobe are presented in Graph I. A total of 851 pituitaries was used in the determination of the curve with the greater number—approxi-



GRAPH I. *Solid line:* anterior-pituitary weights of cockerels in milligrams; *dotted line,* anterior-pituitary weights expressed as percentages of body weight. 851 pituitary glands were used to determine curves.

mately three-fifths—being concentrated in the period from hatching to 40 days of age. The data for weight increase and the increase expressed as a percentage of body weight differ very remarkably. The actual increment is rapid and essentially a straight line up to 30 days of age at which time there is a definite plateau which persists until the 50th day, when the curve again abruptly swings upward through the 90th day. The actual increase is from 0.9 mg. average on day 5, to 8.3 mg. average on day 90. The percentage body-weight, in contrast is at the maximum in the 10th to 13th day period and drops precipitously through the 19th and 24th days. The curve declines more gradually until the 50th day and after that time, although showing minor fluctuations, approaches a plateau.

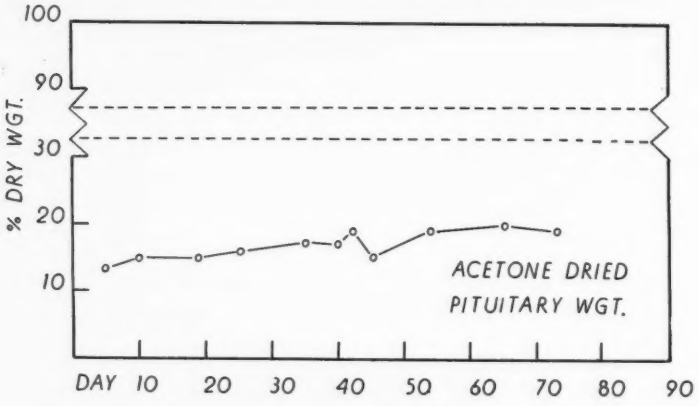
Latimer's (1924) data are interesting for comparison because they show a very gradual decrease which is almost a plateau, for the percentage weights for the whole pituitary after the animals reach about 400 grams of body weight. This would correspond to approximately 50 days in our series, and although his data included the weights of both lobes, there is a fairly good correlation for the form of the curves. Only a limited comparison can be made between the data of Latimer and those of Landauer and Aberle (1935) with respect to percentages for the pituitaries because the latter used only birds 8 months of age. They report a mean pituitary size of 89.5 mg. for cockerels reared under experimental conditions similar to those in our laboratory. This undoubtedly indicates a second plateau after 90 days. The slope of the percentage body-weight curve for the pituitary of older birds given by Latimer is slightly downward and the .0005% shown by Landauer and Aberle is in close agreement with the .0006% which the former reports. These slightly decreasing percentages indicate that the continued increase in body weight after 90 days is not accompanied by relatively the same degree of pituitary increment.

The pituitary weights reported by Juhn and Mitchell (1929) however, do not correlate with these observations. Perhaps, as was suggested by Leonard and Righter (1936), this was due to the fact that their Brown Leghorns were not up to normal weight and the percentages, therefore, were correspondingly high. The birds used by Juhn and Mitchell ranged in age from approximately 8 to 11 months with body weights of about 1000 grams. The pituitary percentages were .0150 for males and .0158 for females. Landauer and Aberle reported a body weight average of 1777 grams at 8 months for White Leghorn cockerels reared indoors and our birds averaged more than 1000 grams at 90 days.

A comparison of Latimer's figures in more detail with those in Graph I indicate that his percentages are somewhat higher than ours at approximately 300 and 400 grams body weight, at which time the pituitaries are observed to be .0018% and .0015% respectively, whereas our data give approximately .0009% and .0008%. It is doubtful if this difference can be explained entirely on the basis of the additional percentage weight contributed by the posterior lobe because at 800 grams body weight Latimer reported pituitary percentages of .0010% whereas our figures for the anterior lobe only show .0009%. There must have been additional differences in the methods employed in the two laboratories.

It is interesting to compare the mammalian data with those of the bird. Stockard (1941) indicated that pituitary size, in proportion to body weight, is relatively greater in smaller breeds of dogs. A similar observation was noted in the data for the Bantam given by Leonard and Righter in which the pituitaries appear to be larger than in the Leghorn with the exception of the data reported by Juhn and Mitchell. Hatai (1914), however, presented a very different picture for the

pituitary of the rat than that given here for the cockerel. His curves, and those reported later by King and Donaldson (1929) indicate that the pituitary weights increase in a straight line when plotted against body weight. These observations are all the more interesting when Rasmussen's (1938) data for human pituitaries are studied. The latter reported that there is a rapid increase in the size of the anterior lobe during childhood, followed by a slower growth until adult weight is reached in the late "teens" which lasts until about 40 years of age, after which there is a very marked decrease in the size of the gland.



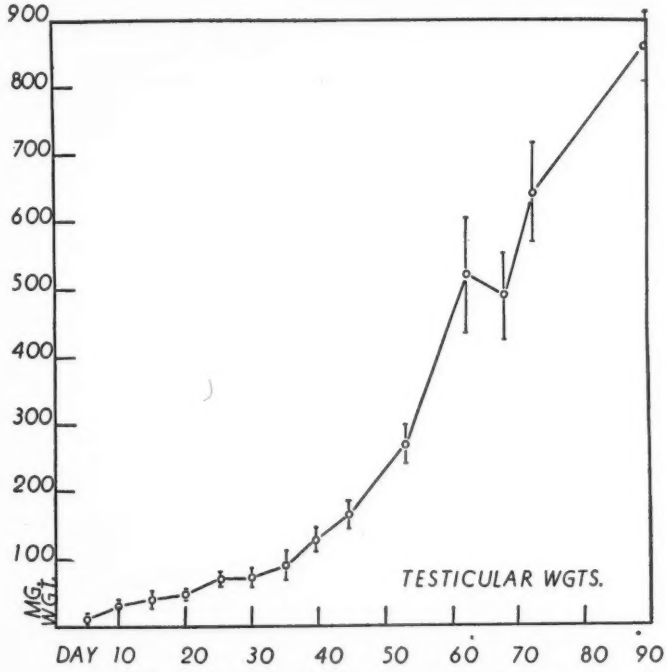
GRAPH II. Percentage of dry weight of anterior-pituitary glands of cockerels after storage in acetone.

This description corresponds more nearly to the composite picture we have described for the fowl than to the data of Hatai for rats. The data of Bates, Riddle and Lahr (1935) for cattle cannot be analyzed in as much detail as the preceding accounts but they do demonstrate 500% to 600% increase in gland size from the veal to the bull or non-pregnant cow.

Acetone-dried pituitary weights. It was important for future assay work to have an analysis of the acetone-dried weights of the pituitaries. These data for pituitaries ranging from the 5th to the 72nd day are shown in Graph II. Percentages range from 13.2% to 18.0% of the fresh gland and the curve shows a very gradual rise with the exception of the fluctuations at 42 and 45 days. The average on the 42nd day is slightly higher than might be expected and is slightly lower on the 45th day and one would probably be justified in projecting the line between these two points. Since the pituitaries were weighed in groups the standard errors are not available; it should only be said that the variations at 62 and 45 days are of doubtful significance.

The actual pituitary weight during this 72-day period increases more than 700% from 0.9 mg. to 6.8 mg. The greatest relative increase in dried weight occurs between the 5th and 10th day when a rise from

13.2% to 15.7% is observed. The fact that the total dry weight increases less than 6% over the entire interval is noteworthy. The amount of solids in the glands, therefore, remains remarkably constant after the 10th day in spite of the great increase in size. Uno (1922) reported that dried pituitary weights for rats from 148 to 185 days of age averages 26.2% of the fresh weight. These percentages are somewhat higher than those of the cockerel, but represent animals of



GRAPH III. Testicular weights in milligrams. 516 testes were used to construct curve.

greater actual and physiological age which may explain part of the difference. Clark (1915) reported that mixtures of given weights of anterior pituitary of fowl with three times their weight milk sugar lose 13.5% upon drying. The data he reported are difficult to analyse but apparently indicate a pituitary weight loss of about 80% which would correspond closely to our figures for older chicks.

Testicular weights. The data for testicular growth are summarized in Graph III which presents the averages for a total of 516 glands. Some statistical features of the curve are interesting especially as regards the standard errors. There is a notable uniformity of growth from hatching to about 55 days of age, at which time the standard errors increase considerably. This period of greater variability in the samples corresponds to the time at which the testes are be-

ginning their greatest relative growth. At 63 days there is another very marked increase in the sample variability and this is well illustrated by the fact that the mean on that day represented an average of 36 testicular weights. Latimer has presented only a small amount of material covering the first 90-day period of development, but his figures do emphasize the considerable variability which occurs especially after rapid growth has begun.

The actual increment of the testes for the various time periods indicates that there is a significant increase in size beginning as early as the 5 to 10-day interval. This gonad size increases at a rather uniform rate until the 30–35 day period at which time there is an abrupt rise in the gonad weights. The slope of the curve is steep and again rather uniform to the 90th day with the exception of the data for the 68th day. The lighter weights of the gonads at this time are probably not overly significant for two reasons: first, the animals were slightly below normal weight; and, second, consideration of the standard errors for the means of the 63rd and 68th days would admit a straightening of the curve during these periods.

It will be noted by reference to Graph I that during the first 30 days there is a relatively greater increase in pituitary size than in the growth of the gonads, but the testicular increment after the 30 to 35 day interval is associated with a plateau in pituitary growth from the 30th to 50th day. After the 50th day, however, the pituitary growth curve rises sharply with a somewhat comparable slope to that for the testicular weights. These observations emphasize the complexity of the relationship of the testes and anterior lobe during the early development of the cockerel and a physiological analysis of this relationship will be discussed in a forthcoming paper.

The early growth of the testes demonstrated by these data suggests a very marked physiological difference between the chicks and the pigeon or ring-dove in their early development. The chick data are comparable to those for the rat as compiled by Donaldson (1926). Donaldson's tables show that at 20.1 grams body weight, testes weights were 0.086 gr.; a 50% increase in body weight was accompanied by nearly a 100% increase in testicular size; and an increase of six times in body weight was associated with more than a 1700% growth of the gonads. These figures demonstrate a relative size increase for the rat testes as great, or greater than that observed for a comparable period in the cockerel. The results also are in agreement with the observations of Smith and Engle (1927) and Clark (1935), that gonadotropins are secreted early by the rat pituitary.

SUMMARY

The anterior lobe of the pituitary of the White Leghorn cockerel increases markedly in size during the interval from hatching to 90 days of age. The weights range from a mean of 0.9 mg. on the 5th day to

8.3 mg. on the 90th day. The increment is not uniform over the entire period, but the growth curve rises sharply for the first 30 days and then develops a plateau during the next 20 days, after which it again swings upward. The data for the pituitary expressed as a percentage of body weight, differ notably from the actual weights. The maximum percentages were observed on the 10th and 19th days following which time the percentage decreased at a very rapid rate. There appears to be a tendency for the curve to plateau in the later stages of the period studied.

Acetone-dried weights of the pituitary range from 13.2% to 18.0% of fresh weight and the greatest relative weight change, an increase of 2.5%, occurs from the 5th to the 10th day after hatching.

The curve for testicular growth during the 90-day post-hatching interval is very different from that for the pituitary. There is a slow but significant increase in weight throughout the first 30 to 35 day period, and this growth begins in the first 5 to 10 day period. No plateau comparable to that observed for the pituitaries was noted but, to the contrary, after the 30–35 day interval there is a very great increase in the growth rate of the testis. Standard errors were low for the series previous to the 35th day but coincidental with the marked increase in testicular size after that day, the standard errors became relatively greater.

It is concluded that the growth of the testes affords evidence of a very early secretory activity by the anterior lobe of the pituitary of cockerels but that this activity is not proportional to the size increase in the gland. A total of 851 pituitaries and 516 testes were used in this study.

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THE GLYCOGENIC ACTIVITY OF CERTAIN CRYSTALLINE STEROIDS OF THE ADRENAL CORTEX WHEN ADMINISTERED SINGLY AND WITH CORTICAL EXTRACT TO FASTED, NORMAL AND ADRENALECTOMIZED RATS[1]

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THE MODIFIED Kendall assay procedure for the evaluation of the glycogenic factors of the adrenal cortex described in the preceding paper (Olson, *et al.*, 1944) has been applied in glycogenic studies of the pure crystalline hormones of the cortex. Reinecke and Kendall (1943) have reported desoxycorticosterone acetate to be totally inactive in glycogenic potency and compounds A, B, and E, i.e., dehydrocorticosterone, corticosterone, and 11-dehydro-17-hydroxycorticosterone to show "less than one-fold difference" in activity. Our results confirm these findings and extend them. By application of our modified procedure to the assay of 11-dehydrocorticosterone, corticosterone, 11-dehydro-17-hydroxycorticosterone, 17-hydroxycorticosterone, allopregnanepentol-3,11,17,20,21, and desoxycorticosterone acetate, certain important differences in both the slope of the regression lines obtained for these steroids and their potency in glycogenic units have been revealed. It has been noted, further, that the administration of mixtures of the C_{11}-oxygen-containing steroids to our assay rats under these same conditions has resulted in measurable augmentations of potency.

I. *The activity of six crystalline hormones of the adrenal cortex in terms of regression line slope and potency.* Six crystalline hormones of the cortex were tested for glycogenic potency in fasted, adrenalectomized rats prefed a high protein diet for four days prior to the fast as outlined in our preceding paper (Olson, *et al.*, 1944). The steroids, with the exception of desoxycorticosterone acetate which was given in 25 per cent alcohol, were administered subcutaneously in 10 per cent alcohol in four equally divided doses over a period of six hours as prescribed in the standard procedure. In order accurately to define the

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slope and position of the regression line in each case, the active compounds were assayed at from six to eight dose levels as shown in Table 1. The regression line constants together with their standard errors as obtained in a statistical analysis of the deposition data by standard methods (Dunn, 1929) are also included in Table 1. The

TABLE 1. GLYCOGEN DEPOSITIONS OBTAINED WITH SIX CRYSTALLINE STEROIDS OF THE ADRENAL CORTEX IN FASTED, ADRENALECTOMIZED RATS

Steroid	Total dose	Rats no.	Mean glycogen deposition	Regression line coefficients†		Significance of differences in b
	mg.		%	$a \pm \sigma_a$§	$b \pm \sigma_b$	t‡
Corticosterone	0.10	8	0.11 ± 0.01*			
	0.19	5	0.16 ± 0.03			
	0.23	7	0.21 ± 0.04			
	0.39	4	0.44 ± 0.08			
	0.47	4	0.52 ± 0.10¶			
	0.58	4	0.84 ± 0.06			
	0.62	3	0.72 ± 0.11			
	0.94	4	1.15 ± 0.09			
	1.15	4	1.40 ± 0.17			
	1.24	4	1.39 ± 0.19	1.24 ± 0.05	2.06 ± 0.29*	0.00
11-Dehydro-corticosterone	0.39	5	0.66 ± 0.09			
	0.42	5	0.47 ± 0.05			
	0.79	6	1.00 ± 0.09			
	0.87	9	1.29 ± 0.08			
	1.16	5	1.40 ± 0.18			
	1.74	7	1.98 ± 0.16	1.37 ± 0.05	2.14 ± 0.21	0.23
11-Dehydro-17-hydroxycorticosterone	0.33	4	0.79 ± 0.13			
	0.39	6	0.61 ± 0.12			
	0.39	5	0.61 ± 0.08			
	0.66	5	1.10 ± 0.15			
	0.77	6	1.05 ± 0.10			
	0.79	5	1.31 ± 0.13			
	1.27	5	1.72 ± 0.16	1.46 ± 0.05	1.91 ± 0.27	0.37
17-Hydroxy-corticosterone	0.44	4	0.73 ± 0.07			
	0.54	4	1.22 ± 0.05			
	0.72	4	1.28 ± 0.14			
	0.88	4	1.80 ± 0.10			
	0.93	6	1.85 ± 0.16			
	1.08	4	2.09 ± 0.17	1.95 ± 0.05	3.29 ± 0.41	2.45
11-Desoxy-corticosterone acetate	1.16	4	0.03 ± 0.01			
	1.01	4	0.02 ± 0.01	0.00	0.00	—
Allopregnane-pentol-3, 11, 17, 20, 21	1.49	4	0.03 ± 0.01	0.00	0.00	—

* The deviations are expressed in terms of the standard error of the mean.

† The regression line constants are for the equation from which glycogen deposition in per cent of the fresh liver weight may be predicted from the logarithm of the dose; i.e., $y = b$ (log dose mg.) $+ a$.

§ The standard errors of the regression line coefficients are calculated according to Dunn (1929).

‡ t is computed according to Burn (1937) and evaluated according to Fisher (1932).

¶ Only those experiments in which the mean glycogen deposition was over 0.45% were used in the calculation of the regression line coefficients.

amount of each steroid to deposit 1.00 per cent liver glycogen in our rats calculated from its regression line formula, the potency of the steroid in glycogenic units per mg., and the significance (Fisher, 1932) of the observed differences are included in Table 2.

In agreement with Reinecke and Kendall (1943), we have found that for all practical purposes the corticosterones, i.e., corticosterone and its 11-dehydro derivative are of identical potency and regression line slope. We have found, on the other hand, that the 17-hydroxycorticosterones, i.e., 17-hydroxycorticosterone and its 11-hydro derivative are markedly different. At a liver glycogen deposition level of 1.00 per cent, 17-hydroxycorticosterone exhibits a regression line

TABLE 2. THE GLYCOGENIC POTENCY OF SIX CRYSTALLINE HORMONES OF THE ADRENAL CORTEX IN TERMS OF CORTICOSTERONE

Steroid	Rats no.	Range of doses	Amount required to deposit 1% liver glycogen	Significance of differences t	Potency in glycogenic U./mg.§
		mg.	μg.		
Corticosterone	23	0.4–1.2	766 ± 39	0.00	1000
11-Dehydrocorticosterone	37	0.4–1.7	671 ± 33	1.86	1143 ± 90†
11-Dehydro-17-OH corticosterone	36	0.4–1.3	574 ± 33	3.77	1335 ± 112
17-Hydroxycorticosterone	26	0.3–1.1	514 ± 19	5.84	1491 ± 92
11-Desoxycorticosterone acetate	8	1.2	INACTIVE		
Allopregnanepentol-3, 11, 17, 20, 21	4	1.5	INACTIVE		

§ A glycogenic unit has been defined by Olson, *et al.* (1944) as the glycogenic activity of one microgram of corticosterone administered to a fasted, adrenalectomized rat in four divided doses at two-hour intervals.

† The standard errors of the deposition equivalents and of the potency ratios are calculated according to the method of Bliss (1939).

having a slope over 1.5 times that of corticosterone and a potency 1.49 ± 0.09 times that of corticosterone. In contrast, 11-dehydro-17-hydroxycorticosterone has a regression line of standard slope and a potency only slightly greater than that of corticosterone at that level of liver glycogen deposition. This is very interesting in view of the fact that the sole difference between the pairs of substances studied, i.e., each C_{11}-hydroxy-steroid and its 11-dehydro derivative, is two hydrogen atoms at C_{11}. It would appear that in the $C_{21}O_5$ series but not in the $C_{21}O_4$ series of cortical steroids, the state of oxidation of the C_{11}-carbon is an important determinant of the glycogenic activity. Wintersteiner's Compound A, an allopregnanepentol containing a completely saturated Ring A was totally inactive at a level of 1.5 mg. as was desoxycorticosterone acetate at a level of 1.2 mg. The relative positions of the regression lines for these compounds are shown graphically in Figure 1.

II. *Augmentation studies in fasted, adrenalectomized rats.* In view of the reports of Kendall (1942) on the apparent enhancement of the activity of the crystalline hormones in natural extracts it became of interest to us to test the effect of various combinations of steroids and extracts upon glycogen deposition in fasted, adrenalectomized rats prepared in accordance with our standard procedure. By adding pure corticosterone ($b = 2.06 \pm .29$) to Extract III ($b = 1.41 \pm .17$) (Olson,

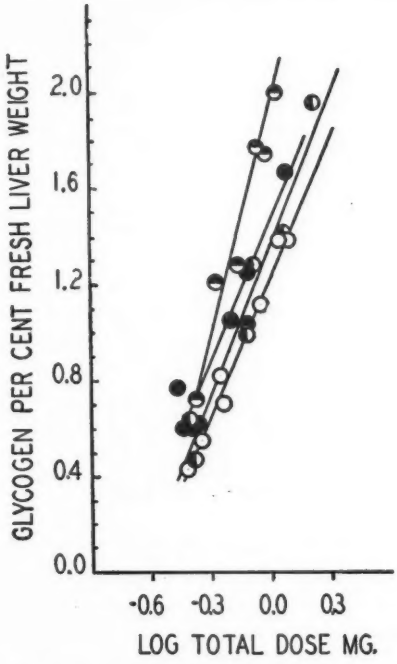

FIG. 1. Logarithm dose-response regression lines for four crystalline steroids of the adrenal cortex. ○ Corticosterone; ◐ 11-Dehydrocorticosterone; ◓ 17-Hydroxycorticosterone; ● 11-Dehydro-17-hydroxycorticosterone.

et al., 1944) the slope of the resulting regression line is raised to that of the predicted line without significant augmentation of potency. The expected responses are calculated in each case by expressing the chosen quantities of extract and steroid in terms of glycogenic units, i.e., micrograms of corticosterone, and then predicting the response by substitution in the standard regression line formula. This evidence is graphically presented in Figure 2. The base line flare in the deposition curve is characteristic of biological responses of this type (Bliss, 1941).

The administration of corticosterone with 17-hydroxycorticos-

terone to our rats, however, resulted in a significant augmentation of glycogenic potency of from 135–175 per cent of that predicted for the components on the basis of their individual glycogen deposition equivalents. We feel that this finding is further proof of a qualitative difference between the C_{11}-oxygen-bearing steroids and extracts having regression lines of lower slope. The simultaneous administration of 11-dehydrocorticosterone and desoxycorticosterone acetate resulted

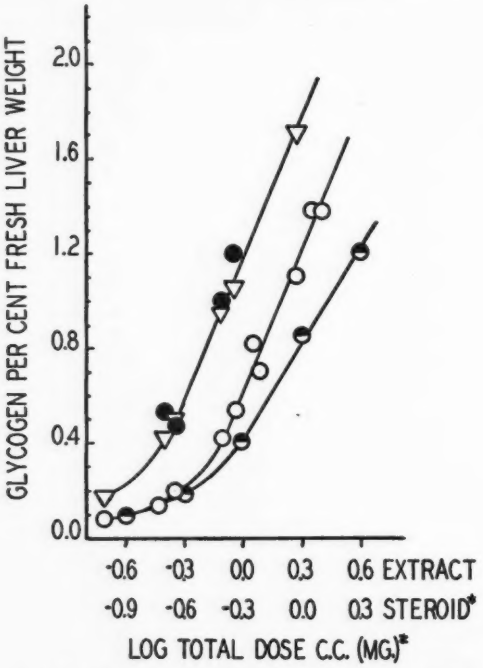

FIG. 2. Logarithm dose-response regression lines for corticosterone and Extract III, separately, and in combination. ○ Corticosterone; ◓ Extract III; ▽ Corticosterone plus Extract III (predicted); ● Corticosterone plus Extract III (found). The upper series of abscissa units refer to the logarithm of the total dose of Extract III in cc.; the lower series refer to the logarithm of the total dose of corticosterone in mg.

in an appreciable change in activity from that predicted for the C_{11}-substituted steroid alone. These data together with an evaluation of the significance of the differences obtained are found in Table 3.

III. *Augmentation studies in fasted, normal rats.* Augmentation studies of a different sort were attempted in the normal rat. Instead of using a cortical extract of low regression line slope as the basal stimulant, it was decided to use the basal secretion of the normal rat's own adrenal cortex in a series of studies. Both Extract II ($b = 1.50 \pm .10$) (Olson, *et al.*, 1944) and crystalline 11-dehydrocorticosterone

were administered in separate experiments to fasted normal rats which had been prefed a high protein and fasted for 24 hours in the same manner as rats prepared for the assay of extracts by the standard procedure.

The basal cortical secretion of the normal rat fed and fasted under these conditions is sufficient to maintain its liver glycogen level at 0.50 ± 0.12 per cent of the fresh liver weight. The adrenalectomized rat prepared under identical conditions shows only a trace of liver

TABLE 3. THE EFFECT OF MIXTURES OF PURE STEROIDS AND ADRENAL CORTICAL EXTRACTS UPON GLYCOGEN DEPOSITION IN FASTED, NORMAL AND ADRENALECTOMIZED RATS

Condition of rat	Rats no.	Materials given	Total doses	Total glycogenic units	Mean glycogen deposition		Significance of differences	Per cent augmentation§
					Found	Predicted		
							t††	
Adrenalectomized	8	Ext. III*	0.46 cc.	460	0.48 ± .09‡	0.50 ± .08	0.31	—
		*B**	0.23 mg.					
Adrenalectomized	8	Ext. III	0.92 cc.	830	1.21 ± .10	1.08 ± .08	1.81	—
		B	0.46 mg.					
Adrenalectomized	4	Ext. III	0.47 cc.	400	0.54 ± .10	0.44 ± .10	0.71	—
		B	0.18 mg.					
Adrenalectomized	4	Ext. III	0.93 cc.	730	1.00 ± .09	0.96 ± .10	0.30	—
		B	0.36 mg.					
Adrenalectomized	7	*F*	0.22 mg.	400	1.05 ± .10	0.44 ± .09	4.38	+74
		B	0.18 mg.					
Adrenalectomized	6	*F*	0.44 mg.	970	1.54 ± .11	1.21 ± .09	2.33	+35
		B	0.36 mg.					
Adrenalectomized	4	DCA	1.51 mg.	370	0.30 ± .07	0.40 ± .09	0.83	—
		A	0.37 mg.					
Normal	4	Ext. II	0.5 cc.	650	0.56 ± .04	0.85 ± .13	2.14	−28
	4		1.0 cc.	770	0.52 ± .10	1.01 ± .13	2.98	−42
	4		2.0 cc.	1050	0.94 ± .10	1.28 ± .13	2.07	−32
Normal	4	*A*	0.21 mg.	660	0.92 ± .25	0.87 ± .14	0.17	—
	4		0.42 mg.	870	1.65 ± .20	1.09 ± .14	2.28	+91

* The extracts used in these experiments retain the numeral designations given them in the preceding paper (Olson, *et al.*, 1944). The steroids are named according to the alphabetical sequence of Kendall (1943), i.e., Compound *B* is corticosterone, *F* is 17-hydroxycorticosterone and *A* is 11-dehydrocorticosterone. DCA is desoxycorticosterone acetate.

† t, the significance of the difference in the observed and predicted glycogen depositions is calculated according to Burn (1937) and evaluated according to Fisher (1932).

‡ The recorded deviations are the standard errors of the mean calculated for small groups according to Burn (1937).

§ Per cent augmentation is calculated in the following manner:

$$\text{Per cent augmentation} = 100 \text{ antilog} \left[\frac{\text{Deposition found} - \text{Deposition predicted} - 1}{\text{Slope predicted line}} \right].$$

glycogen (0.032 ± 0.003 per cent) (Olson, *et al.*, 1944) under fasting conditions but responds to cortical therapy by depositing liver glycogen in proportion to the logarithm of the dose of hormone given. Although it is generally hazardous to transpose data from deficient to normal animals in making calculations of the rate of an endocrine secretion, an approximation to the truth is often obtained by such a step. If one assumes, for the sake of the approximation, that the effect of corticosterone injected subcutaneously in divided doses is comparable to that of the natural cortical secretion, one may gain an idea of the order of magnitude of the rate of secretion by consulting the stand-

ard dose-response curve for corticosterone. By substitution in the equation for the regression line of corticosterone (Table 1) it may be found that a glycogen level of 0.50 ± 0.12 per cent is equivalent to the activity of 438 ± 37 μg. of corticosterone over a period of eight hours. The glycogenic response in our adrenalectomized rats has been found to persist undiminished for four hours beyond the eight hour assay period. In view of these considerations and without regard for a possible action of adrenaline, it is conceivable that the adrenal cortex of a normal rat on a high protein dietary regime secretes an amount of C_{11}-oxygen-bearing steroids equivalent to 876 ± 74 glycogenic units per day. That the survival of young adrenalectomized rats can occur without the aid of the C_{11}-oxygen-bearing steroids (Grollman, 1934) and with smaller doses of corticosterone when that steroid is given alone (Kuizenga, *et al.*, 1940) is recognized. In all probability our calculated rate of secretion is a maximal daily value for the C_{11}-oxidized hormones and in view of possible synergisms, uninformative about the actual daily rate in mg.

If normal fasting rats prefed high protein diets are treated with 0.5, 1.0, and 2.0 cc. of Extract II, equivalent respectively to 200, 320, and 600 glycogenic units, there is less glycogen in the liver at the end of the assay period than would be predicted from the total unitage supplied by the animal's glands and the injected volumes of extract. This loss of apparent activity of 28–42 per cent is accompanied by a decline in the slope of the resulting regression line to approximately 1.0. If, however, normal fasted rats are injected with crystalline 11-dehydrocorticosterone there is a sizeable augmentation and an improvement in the slope (2.8) of the resulting regression line. A summary of these experiments is included in Table 3.

DISCUSSION

There is now a wealth of evidence (Long, *et al.*, 1940; Grattan and Jensen, 1940; Ingle, 1940; Reinecke and Kendall, 1943) to indicate that neither those steroids which are not ketonic at C_3 and α, β-unsaturated in Ring A nor those which are without an oxygen substituent at C_{11} have any influence upon carbohydrate and protein metabolism in either the intact or adrenalectomized animal. Little, however, has been done to differentiate qualitative and quantitative differences in the physiological properties of the individual C_{11}-oxygen-substituted, C_3-ketonic steroids. Our assays show that while corticosterone and its 11-dehydro derivative are of comparable potency in inducing glycogen deposition in the fasted, adrenalectomized rat, 17-hydroxycorticosterone and its 11-dehydro derivative are markedly different. Ingle (1943) has found 17-hydroxycorticosterone to be superior to other C_{11}-oxygen-substituted steroids of the adrenal cortex in promoting work tolerance in adrenalectomized, nephrectomized rats (Ingle, 1944). 17-Hydroxycorticosterone has also been reported

by Grattan and Jensen (1940) to be more active than corticosterone in preventing insulin hypoglycemia and in storing liver glycogen in young mice.

Kendall (1942) has recently commented upon the augmentatory effect evidenced by crude extracts of adrenal cortical principles when their assay values are compared with those of their solid-equivalents of the pure crystalline steroids. By the partition of a natural mixture of hormones between immiscible solvents, he was able to destroy this enhancement which he could not then restore by recombination of the solids from both phases. By combination of corticosterone and 17-hydroxycorticosterone in amounts of approximately 0.2 mg. each, we have been able to augment their glycogenic potency 75 per cent. It is possible that this augmentation accounts for part of the calculated 300–400 per cent enhancement found in natural extracts.

It has already been pointed out (Olson, *et al.*, 1944) that qualitative differences in the activity of extracts prepared by different methods exist, and that the slope of the regression line plotted from the assay results is a fairly good criterion of that difference. Further evidence for qualitative differences in the action of certain prepared extracts and of the crystalline C_{11}-oxygen-substituted steroids is found in augmentation studies in fasted, normal and adrenalectomized rats. In the adrenalectomized rat, the simultaneous administration of extracts of low regression line slope and corticosterone resulted in a quantitative recovery of the injected unitage. In the normal rat, the injection of an extract of low regression line slope alone resulted in incomplete recovery of the unitage while the injection of 11-dehydrocorticosterone gave an augmented recovery of 191 per cent at a level of 0.4 mg. of steroid. We interpret these differences to indicate a relative deficiency of C_{11}-oxygen-substituted steroids in cortical extracts having regression lines of low slope.

SUMMARY

The glycogenic activity of six crystalline hormones of the adrenal cortex has been characterized in terms of regression line slope and quantitative potency.

17-Hydroxycorticosterone has been found to be the most active while desoxycorticosterone acetate and allopregnanepentol-3,11,17,-20,21 were found to be totally inactive.

Simultaneous administration of corticosterone and 17-hydroxycorticosterone to our assay rats resulted in an augmentation of potency.

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FURTHER OBSERVATIONS ON INDUCED OVULATION IN MICE. A REFRACTORY PERIOD IN EARLY PREGNANCY

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IN AN EARLIER REPORT, it was shown that ovulation was induced in mice within 18 hrs. after a single subcutaneous injection of a chorionic gonadotrophin (Burdick, Watson, Ciampa and Ciampa, 1943). During the experiment with mated mice it was soon discovered that although ovulation could be regularly induced in animals having implanted embryos, negative results were usually obtained in those animals during the first few days while the fertilized ova were being transported to the uterus. This present investigation was undertaken to collect more data on the duration of this refractory period.

PROCEDURE

Mated mice were selected each morning after ascertaining the presence of the vaginal plug. Single injections of a chorionic gonadotrophin[1] were given on different days and in various amounts as indicated in table 1. Injections of 5 I.U. or more were considered adequate for this investigation, since it had been found earlier that 0.7 I.U. was a minimal ovulating dose for mice in later pregnancy. The reproductive tracts were examined by the method outlined previously (Burdick and Whitney, 1941). If fertilized ova were not found in the oviducts, especially on the fourth day after mating, the uterus was flushed with saline solution and the fluid examined for blastocysts. These mice were killed 18 to 24 hrs. after they were injected.

RESULTS

Relationship of stage of embryos to ovarian response. The stage in development shown by the embryos at the time of autopsy gives us a rather definite idea of the lapse of time since normal ovulation and fertilization. Table 1 shows the response of mouse ovaries to various amounts of the chorionic gonadotrophin, arranged according to the age of the fertilized ova. The hours indicated follow the schedule given by Lewis and Wright (1935). During the first 36 hrs. while the ova are in 1- and 2-celled stages, only 2 of 16 mice ovulated. A total of

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[1] Generous supplies of the chorionic gonadotrophin, Follutein were furnished by E. R. Squibb & Sons, New Brunswick, New Jersey.

TABLE 1. DURATION OF A REFRACTORY PERIOD IN EARLY PREGNANCY

Animal no.	Amount of injection in I.U.	Number of embryos	Number of ova in ampullae
	1-celled eggs: 0 to 24 hrs.*		
45	10	5	0
46	15	4	0
	2-celled eggs: 24 to 38 hrs.		
19	5	8	0
22	5	7	0
47	5	7	0
24	5	6	0
43	10	9	0
66	10	7	0
67	10	7	0
84	10	10	1
85	15	7	0
65	20	9	0
75	20	9	4
76	30	8	0
77	40	9	0
73	50	9	0
	4- to 9-celled eggs: 38 to 64 hrs.		
51	5	10	13
55	5	9	0
59	10	8	0
54	10	12	6
1	20	11	4
97	20	8	0
74	50	5+	0
	Morulae in intramural region: 60 to 70 hrs.		
60	10	8	0
61	10	7	3
99	10	7	0
100	10	7	0
79	15	7	0
101	15	4	8
98	20	8	0
	Blastocysts free in uterus		
50	5	6	1
80	5	not counted	9
30	10	7	10
56	10	6	3
57	10	6	12
58	10	not counted	9

* Time schedule from Lewis and Wright (1935).

only 5 o/a with granulosa cells was found in the ampullae of these 16 animals.

In the mice with 4- to 9-celled ova, 24 to 36 hrs. after fertilization, 3 of the 7 had 13.6, and 4 ova respectively. Possibly mouse no. 51 with 13 ova should have been placed in the next group, since 61 hrs. had elapsed since finding the vaginal plug. The ova, however, were only 6- to 8-celled which might indicate either a delayed ovulation or late fertilization.

Ovaries of mice with embryos in the morula stage in the intramural region of the oviduct between 60 and 70 hrs. are also not

particularly responsive. Only 2 of 7 animals in this group ovulated, one having 3 and the other 8 ova.

A quite different result was obtained by the time the embryos had developed into blastocysts lying free in the uterine lumen. These blastocysts were flushed from uteri 78 to 120 hrs. after the vaginal plugs had been found. All 6 of the animals responded to the gonadotrophin with a total of 44 ova. Mouse no. 57 had blastocysts within intact zonae in the uterus at 81 hrs., but another animal, not shown in the table, with early blastocysts still in the intramural region, had not ovulated again although the ova were estimated to be 83 hrs. old. Thus it is seen that the mouse ovary is relatively refractory to the action of chorionic gonadotrophin during the first three days of pregnancy. Ovulation is more regularly induced by the time the blastocysts are well developed and in the uterus. This is approximately 78 hrs. after normal ovulation.

Effect of dosage on induced ovulation in early pregnancy. Inasmuch as 5 I.U. of the chorionic gonadotrophin was known to be more than necessary to induce ovulation in mice after embryos had become implanted in the uterus (Burdick, *et al.*, 1943), it was thought that the ovaries of mice in early pregnancy might be more responsive to larger doses of the gonadotrophin. This did not prove to be true since injections of 15 to 50 I.U. were no more effective than 5 I.U. Whether or not smaller doses would have been equally effective is not shown in this experiment.

DISCUSSION

From the above data it is evident that the mouse ovary is relatively refractory to the action of chorionic gonadotrophin during the first 3 days of pregnancy. At no time is an absolutely refractory condition found in all animals, but the first 36 hrs. after ovulation closely approximates that condition. Snyder and Wislocki (1931) found that there was a refractory period of 4 days after normal ovulation in the pregnant and pseudo-pregnant rabbit.

They, too, found some variation and decided that the response was dependent upon dosage. An examination of their report, however, shows irregularities in response with even the highest doses. Jares (1932) reported that ovulation could be induced repeatedly in the rabbit, ". . . whenever desired, by successive injections of pregnancy urine, providing the dose was large enough." An examination of his data, however, shows no instance of a second injection occurring earlier than 3 days after the first injection. Only one animal injected again after 3 days had a ruptured follicle. All of the other rabbits received the second injection 4 to 10 days later, which would place the induced ovulations after the refractory period.

Jares also showed that about 3 times as much pregnancy urine was needed to induce ovulation in the presence of corpora lutea as in

the normal estrous rabbit. He suggested that the corpora lutea exerted an inhibitory influence, but he also pointed out the possibility that the ovary may not have any matured follicles after a rather complete ovulation. Engle (1939) maintains that the chorionic gonadotrophin does not act directly on the ovary and can induce ovulation only through the mediation of the animal's own pituitary. Furthermore, only the normally ripened follicles will rupture. According to this view, then, the refractory period would be primarily due to a depletion of activating substances of the pituitary at the time of the normal ovulation or to a refractory condition of that gland.

SUMMARY

In an earlier report it had been shown that single subcutaneous injections of chorionic gonadotrophin regularly induce ovulation in pregnant mice after the embyros had implanted in the uterus. This investigation shows that the mouse ovary is relatively refractory to this gonadotrophin during the first 3 days of pregnancy while the ova are passing through the oviducts. Only 2 of 16 mice ovulated during the first 36 hrs. of pregnancy, and only 5 of 14 animals ovulated again during the 38th to 70th hrs. of pregnancy. Ovulation is regularly induced when blastocysts are well developed and lie free in the uterine lumen on the fourth day.

Large doses of chorionic gonadotrophin are apparently no more effective in causing the second ovulation than 5 I.U., the lowest amount used.

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HEPATIC LESIONS AND EXPERIMENTAL HYPERTHYROIDISM

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VARIOUS AUTHORS have reported hepatic changes in hyperthyroid animals. Farrant, in 1913, reported that the liver of thyroid-fed rabbits and cats showed fatty degeneration which was most marked around the center of the lobule. Hashimoto (1921) found hepatic changes in rats fed thyroid gland. He observed central parenchymatous degeneration in the liver lobules which varied from simple fatty degeneration to necrosis. Chronic passive congestion about the central vein, and an increase in mitotic figures about the periphery were also observed. Gerlei (1933) studied the liver of rabbits injected with thyroxin and observed the occurrence of extensive degeneration and necrosis. The pathological changes in the liver during Graves' disease have been reviewed by Frazier and Brown (1935), Boyce (1940) and McIver (1942).

Other studies have shown that the requirements for the B vitamins are increased during experimental hyperthyroidism (Himwich, Goldfarb and Cowgill, 1932; Drill, 1943). Further, the loss in weight produced by toxic doses of thyroid gland can be prevented by administering vitamin B, and yeast concentrate in both rats (Drill and Sherwood, 1938; Drill, Overman and Leathem, 1943) and in dogs (Drill and Shaffer, 1942).

In the earlier studies on the effect of thyroxin and thyroid gland on the liver the increased requirements of the B vitamins were not recognized. The possibility remained that the hepatic changes reported may be due both to a loss of weight and the effect of the thyroid feeding. As a large supplement of the B vitamins will prevent a loss of weight in thyroid-fed rats, it seemed of interest to determine if thyroid gland would still produce hepatic changes when the food intake and weight were maintained by such therapy.

Methods. Adult white rats were used as the experimental animal. Desiccated thyroid gland, containing 0.213% iodine was fed orally each day. The female rats received 100 mgm. of thyroid gland per day for the length of time indicated in the tables. This dose of thyroid is generally toxic enough to produce a decrease in the body weight of rats receiving a diet containing a small but ordinarily adequate amount of the B vitamins. If the diet of such animals is supplemented with a rich source of the vitamin B complex they will

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regain their lost weight. Male rats are more sensitive to the same dose of thyroid gland than female rats, and will not regain their lost weight if large doses of the B vitamins are administered (Drill, 1938). They will, however, regain their lost weight under therapy when 50 mgm. of thyroid gland is fed per day. The male rats receiving this smaller dose of thyroid gland are noted in the tables.

All rats received a synthetic diet consisting of: casein, 20; Crisco, 10; cod-

TABLE 1. HEPATIC CHANGES OF NORMAL RATS ON A NORMAL SYNTHETIC DIET

Rat no.	Sex	Days fed synthetic diet	Change in weight grams	Liver pathology
1	F	81	+15	Normal
2	F	64	+39	Very slight changes seen. Cells slightly swollen, sinusoids compressed, cytoplasm finely granular.
3	F	81	+43	Normal
4	F	81	+21	Normal
5	F	78	+16	Normal
6	F	78	+64	Distinct lesions, Marked vacuolation, presumably fatty changes, involving principally periphery of lobules. More than 50% of liver cells contain large clear vacuoles (signet ring forms).
7	F	78	+ 2	Normal
8	F	78	+31	Normal
9	F	78	+48	Normal
10	F	78	+31	Very slight vacuolation.
11	F	78	+25	Normal
12	M	16	+12	Normal
13	M	66	+37	Normal
Normal rats injected with 500 gamma of thiamine per day for 44 days				
14	F	44	+ 6	Normal
15	F	44	+ 5	Normal
16	F	44	+12	Normal
17	F	44	+32	Normal
18	F	44	+15	Normal

liver oil (U.S.P.), 4; salts, 4; and starch 62 parts. Supplements of the B vitamins were given as indicated for each group. Sections of liver of each rat were fixed in Bouin's solution and stained with hematoxylin and eosin, a total of 68 animals being used.

RESULTS

Normal rats. As normal rats may show hepatic changes, 13 normal rats were killed and the liver examined as controls. These animals received the synthetic diet for the time indicated in Table 1, together with a daily supplement of 200 mgm. of a dried baker's yeast which contained 23 I.U. of vitamin B_1, 60 gamma of riboflavin, 85 gamma of pyridoxine and 120 to 150 gamma of calcium pantothenate per gram. This amount of yeast will allow a gain in weight in normal animals, but as the rats used were adult, there was little change in weight. As some of the thyroid fed rats were injected with thiamine, five control

rats were also injected for 44 days. Of the 18 normal rats one showed distinct hepatic lesions consisting of a marked vacuolation involving more than 50% of the cells, which were presumably fatty changes. Two of the rats showed very slight changes from the normal (table 1).

Thyroid-fed controls. Twenty rats received the same diet and yeast supplement as did the normal rats, plus 100 mgm. of thyroid gland orally per day (table 2). There was a marked and steady loss of

TABLE 2. THYROID-FED CONTROLS RECEIVING 100 MGM. DESICCATED THYROID GLAND PER DAY

Rat no.	Sex	Days fed synthetic diet and thyroid gland	Change in weight grams	Liver pathology
19	F	17	−25	Normal
20	F	33	−61	Slight fatty changes (vacuolization) around the central vein; confined to central zone.
21	F	33	−60	Vacuolization; more severe in central and intermediate zone.
22	F	51	−77	Normal
23	F	51	−82	Normal
24	F	47	−47	Normal
25	F	47	−66	Normal
26	F	47	−66	Normal
27	F	47	−82	Normal
28	F	47	−30	Normal
29	F	47	−34	Normal
30	F	78	−19	Normal
31	F	78	−13	Normal
32	M	45	−178	Normal
33	M	68	−30	Normal
34	M	17	−39	Slight granular degeneration. Hyperchromatism of some nuclei.
35	M	17	−40	Normal
36	M	17	−40	Slight fatty degeneration (?)
37[1]	M	34	−45	Normal
38[1]	M	34	−153	Normal

[1] Fed 50 mgm. of thyroid gland per day.

weight in these animals. The striking point was that the livers, aside from histological evidence of glycogen depletion, were entirely normal. Minor differences were seen, but similar changes were noted also in normal animals, and in these animals the changes are probably due only to the glycogen depletion. One rat showed a slight granular degeneration with hyperchromatism of some of the nuclei of hepatic cells. Slight fatty degeneration was noted in three other rats. The remaining 16 animals did not present any significant changes from normal, and in none of the animals was there any necrosis or hemorrhage in spite of the marked loss of weight that occurred.

Thyroid-fed rats receiving vitamin supplements. Eleven rats received the same basal synthetic diet and yeast supplement as the normal animals plus thyroid gland for 57 or 81 days (table 3). They were continued on this regimen for 26 days during which time they

TABLE 3. HYPERTHYROID RATS RECEIVING 100 MGM. OF THYROID GLAND PER DAY PLUS SUPPLEMENTS OF VITAMIN B_1 AND YEAST

Rat no.	Sex	Days fed synthetic diet	Change in weight grams	Liver pathology
39	F	81	+10	Normal
40	F	81	+28	Mild granular degeneration
41	F	81	−35	Normal
42	F	81	+ 5	Mild granular degeneration
43	F	81	− 5	Mild granular degeneration
44	F	81	+38	Mild granular degeneration
45	F	81	+28	Normal
46	F	81	+24	Normal
47[1]	M	57	+ 3	Slight fatty degeneration
48[1]	M	57	− 5	Normal, except for hemorrhages (probably artifacts).
49[1]	M	57	0	Normal

[1] Fed 50 mgm. of thyroid gland per day.

lost an average of 24 grams in weight. On the 26th day a daily supplement of 1.0 gram of yeast was administered, which contained 50 I. U. of vitamin B_1, 80 gamma of riboflavin, 30 gamma of pyridoxine, 130 gamma of calcium pantothenate and 600 gamma of niacin per gram. One-half of a milligram (500 gamma) of thiamine was also injected subcutaneously per day. With these supplements the animals began to regain their lost weight, which in most cases had been entirely recovered when the animals were autopsied (table 3). Five of the rats showed hepatic lesions consisting of a mild granular or fatty degeneration. One rat showed hemorrhages in the sections of liver but was otherwise normal.

Nineteen animals were also fed the basal diet plus 100 mgm. of thyroid gland per day for 77 days (table 4). At the start of thyroid

TABLE 4. HYPERTHYROID RATS RECEIVING 100 MGM. OF THYROID GLAND PER DAY PLUS A DAILY YEAST SUPPLEMENT

Rat no.	Sex	Days fed synthetic diet	Change in weight grams	Liver pathology
50	F	77	+ 2	Normal
51	F	77	−21	Normal
52	F	77	0	Normal
53	F	77	+15	Normal
54	F	77	+23	Normal
55	F	77	+17	Normal
56	F	77	+ 1	Normal
57	F	77	+11	Normal
58	F	77	+ 7	Normal
59	F	77	+34	Normal
60	F	77	−12	Normal
61	F	77	+ 9	Normal
62	F	77	+17	Normal
63	F	77	−11	Normal
64	F	77	− 1	Normal
65	F	77	+16	Normal
66	F	77	+36	Normal
67	F	77	+19	Normal
68	F	77	+19	Normal

feeding they also received 1.0 grams of yeast concentrate per day which supplied 200 I. U. of vitamin B_1, 230 gamma of riboflavin, 200 gamma of pyridoxine and 1.5 to 2.0 mgms. of calcium pantothenate. This supplement was sufficient to prevent a loss of body weight in most of the animals. Sections of livers of these animals did not show any significant change from normal.

DISCUSSION

Our rats fed thyroid gland for 17 to 78 days did not show any striking change in hepatic structure, even though the animals had lost from 13 to 178 grams in body weight during this period (table 2). The amount of B vitamins supplied in the diet of these rats is adequate for normal animals, but is increased during hyperthyroidism so that a relative deficiency ensues (Drill, 1943). Although some slight changes were found in 4 of the thyroid-fed rats, they were not any greater than might be observed in control rats. By supplementing the diet of the other groups with a rich source of the vitamin B complex the body weight was generally maintained permitting thyroid gland to be fed for a longer period of time. The livers of these rats also were histologically normal.

Farrant (1913) observed a fatty degeneration in the liver of thyroid-fed cats and rabbits which was most marked around the center of the lobules. His animals lost from 28 to 35% of their body weight. Hashimoto (1921) also observed hepatic changes varying from simple fatty degeneration to necrosis in rats fed 100 to 500 mgm. of thyroid gland per day. Both the thyroid dose and the per cent of iodine present (0.488 per cent iodine) were higher than in the present experiment, and most of his animals died in two to fourteen days. His animals received a diet of bread and milk, with green vegetables on alternate days. One wonders whether the laboratory diets in use at that time, together with the thyroid feeding, were the actual cause of the hepatic lesions. Current studies have clearly shown that a lack of dietary protein per se may produce liver damage. Also, the dose of thyroid used by Hashimoto was especially toxic. Gerlei (1933) studied the effect of daily subcutaneous injections of 4.0 mgm. of thyroxin in rabbits. The animals died in 4 to 5 days and the livers showed extensive degeneration and necrosis.

Haben (1935), in his study on the effect of thyroxin on the liver, only noted important hepatic changes when an intercurrent infection occurred. More recently McIver and Winter (1943), in connection with the effect of anoxia on the liver during hyperthyroidism, studied the effect of thyroxin on the liver of 31 control rats. The animals received 0.1 mgm. of thyroxin daily for two to three weeks. Without exception no notable histological changes were observed. Some minor changes were present in the thyroxin-treated rats, the cells appearing more homogenous and compact, this being ascribed to a probable de-

pletion of liver glycogen. They also had 12 hyperthyroid rats that died from intercurrent infections, and when the livers of 10 of these animals were examined, four showed degenerative changes. This latter point would seem to fit in with the observations of Haben. Our results confirmed the observation of McIver and Winter, in that no notable histological changes were found in the liver of hyperthyroid rats. McIver and Winter point out that the acute hepatic lesions observed in patients dying of hyperthyroidism, may be due to anoxia, especially when pulmonary edema and other complications appear, or to anoxia produced by the hyperpyrexia of thyroid crisis.

In a view of these studies it seems that the earlier observations reporting hepatic damage in experimental hyperthyroidism may be due to the use of imbalanced diets. These experiments were duplicated by feeding a small but normal amount of yeast to the thyroid-fed animals, so that a loss of weight still occurred. With the synthetic diet used no significant hepatic changes were noted even after a marked loss of weight. Preventing the loss of weight by the administrations of the B complex supplements allowed the thyroid to be fed for a longer period of time. Even then no histological changes were noted in the liver.

SUMMARY

Rats fed sufficient thyroid gland to produce a marked loss of weight over a period of 17 to 78 days did not show any significant changes in the liver when compared to normal animals on the same diet. Other animals, receiving a daily supplement of years concentrate to prevent a loss of body weight, also showed a histologically normal hepatic structure, even when thyroid gland was fed for 81 days.

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THE EFFECT OF DIETHYLSTILBESTROL ON THE IN VITRO FORMATION OF PHOSPHOLIPIDS IN THE LIVER AS MEASURED WITH RADIOACTIVE PHOSPHORUS

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LAWRENCE and Riddle (1916) reported that lipids of the blood are increased in the fowl during the egg-laying season. This finding was confirmed by Lorenz, Entenman and Chaikoff (1938), who showed that in birds actively engaged in egg-laying there is a marked but variable increase in the levels of triglycerides, phospholipids and free cholesterol of the blood. Since the rise in blood lipids was observed first in the pubescent female containing ova in the rapid growth stage, but at no time from early age to past maturity was detectable in the male bird, these workers concluded that ovarian activity is involved in these lipid changes. This conclusion was upheld in later experiments (Entenman, Lorenz and Chaikoff, 1938) in which it was shown that injections of pregnant-mare serum, which contains a gonad-stimulating principle, led to a rise in the levels of all the blood-lipid constituents of the immature female bird. Further investigations showed that injections of ovarian hormones brought about similar rises in all blood-lipid constituents not only in the female bird but in the male bird as well (Lorenz, Chaikoff and Entenman, 1938). This observation has been confirmed by several investigators (Zondek and Marx, 1939; Landauer, Pfeiffer, Gardner and Man, 1939; Riddle and Senum, 1939). Both natural and synthetic estrogens have now been shown to be effective (Entenman, Lorenz and Chaikoff, 1940; Flock and Bollman, 1942).

In a previous report it was demonstrated with the aid of radioactive phosphorus as a labeling agent that surviving slices of liver suspended in a Ringer medium can synthesize phospholipid (Taurog, Chaikoff and Perlman, 1942). This procedure has been used here to compare the phospholipid activities of livers excised from normal birds and from birds that had been injected with diethylstilbestrol.

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It was found that the *in vitro* capacity of the liver to convert inorganic phosphorus to phospholipid is increased by the injection of stilbestrol.

METHODS

The birds recorded in Table 1 were immature, male, single-comb, white Leghorns of the Poultry Division stock. In each experiment, 2 birds of approximately the same age and weight were used. One was injected intramuscularly with 10–15 mg. of diethylstilbestrol dissolved in 0.2 cc. of Wesson Oil. The other bird, serving as a control, received 0.2 cc. of Wesson Oil intramuscularly.

The birds were sacrificed at varying intervals after the injections and their livers rapidly removed and weighed. Liver slices were made by hand with a single-edge razor blade; the same portion of the liver was used throughout all experiments for the preparation of these slices. The slices were collected in a Petri dish containing an oxygenated bicarbonate-Ringer solution prepared as described by Krebs and Henseleit (1932). Approximately 600 mg. (wet weight) of liver slices were prepared; 2 portions, each weighing 250–300 mg., were then weighed out rapidly on pieces of cellophane. One portion was transferred to a weighed watch glass and dried to constant weight at 85° for a dry-weight determination. The other portion was transferred to a 50 cc Erlenmeyer flask containing 5 cc. of the bicarbonate-Ringer solution to which had been added approximately 0.25 microcuries of radioactive phosphorus (P^{32}) as Na_2HPO_4. The preparation of this radioactive Ringer's solution and the measurement of newly formed phospholipid have been described elsewhere (Taurog, Chaikoff and Perlman, 1942).

Blood was removed from the wing vein just before the birds were sacrificed for the determination of lipids. Total fatty acids were measured by an oxidative procedure described elsewhere (Chaikoff and Kaplan, 1934). The P of phospholipids was determined by King's method (1932). The factor 25 was used to convert mg. of lipid phosphorus to mg. of phospholipid.

RESULTS

The effects of a single injection of 10–15 mg. of diethylstilbestrol on the concentration of phospholipids and total fatty acids of whole blood are recorded in Table 1. An increase in the level of phospholipids was observed as early as 6 hours after the injection. Increases in total fatty acids as well as phospholipids were found at later intervals, namely 10.5 to 16 hours.

The total fatty acid content of the livers of the birds are also recorded in Table 1. No significant rise was noted as early as 6 hours. The first definite evidence of an increase was obtained 12–13 hours after the injection of the diethylstilbestrol and by the time 15–16 hours had elapsed total fatty acids of the liver had risen from approximately 4 to 9–10 per cent.

As noted above, the 2 birds used in each experiment (the stilbestrol-injected and the control) were closely matched with respect to their ages and weights. The preparation of liver slices from both birds and their incubation in the radioactive Ringer's medium were carried

out at the same time in order to ensure identical treatment for both livers during this part of the experiment.

The percentages of the Ringer's P^{32} recovered as phospholipid have been calculated on the basis of wet weight as well as dry weight of liver. The values obtained in 15 experiments are recorded in the last 2 columns of Table 1. The rate of incorporation of Ringer's P^{32} into

TABLE 1

Expt. no.	Interval after injection	Control or injected[1]	Bird weight	Liver weight	Liver total fatty acids	Whole blood lipids		Per cent of Ringer's P^{32} converted to phospholipid by liver slices	
						Phospholipids	Total fatty acids	per gm. wet wt.	per gm. dry wt.
	hours		*gm.*	*gm.*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*		
1	6	control	964	23.9		250	440	4.7	29
		injected	951	18.8		330	420	6.2	38
2	6	control	872	21.0		200	308	4.3	25
		injected	1011	21.7		320	540	6.9	40
3	6	control	1930	41.8	4.8	292	415	3.6	19
		injected	2000	42.5	6.2	369	503	4.0	22
4	6	control	1820	29.8	3.6	269	374	2.1	12
		injected	1880	35.0	4.2	293	394	3.1	16
5	6	control	790	19.3	3.3	288	545	4.5	24
		injected	820	18.5	3.6	332	605	4.9	27
6	6	control	770	24.3	4.0	300	615	4.6	25
		injected	770	20.2	4.2	315	610	6.6	36
7	10.5	control	960	25.3	3.2	303	477	5.4	28
		injected	950	24.4	4.1	363	600	7.6	40
8	12	control	961	19.7		210	385	3.8	25
		injected	953	23.9		400	800	6.8	40
9	12	control	990	26.3	4.1	297	440	5.5	29
		injected	990	25.2	6.0	447	753	5.5	29
10	12.5	control	750	16.0	3.4	309	520	3.7	21
		injected	750	18.7	5.9	496	1010	3.9	22
11	12.5	control	840	15.5	3.5	278	485	2.6	14
		injected	840	24.2	3.9	443	1030	7.1	40
12	13	control	970	23.8	3.5	336	500	3.3	22
		injected	970	26.8	8.2	425	765	7.4	45
13	13.5	control	991	20.5		270	433	5.0	26
		injected	796	16.9		400	825	6.2	33
14	15	control	990	26.0	3.8	357	520	4.6	24
		injected	980	28.4	9.1	500	915	7.7	41
15	16	control	970	27.4	3.7	316	477	5.2	25
		injected	970	32.6	10.3	527	950	7.9	39

[1] Each injected bird received 10 mg. of diethylstilbestrol except in experiments 3 and 4. In the latter experiments 15 mg. of diethylstilbestrol were injected.

phospholipid may be considered a measure of the rate of phospholipid formation by the slice. The results show that the *in vitro* formation of phospholipid is greater in the liver slice of the stilbestrol-treated bird than in that of the bird that had received no stilbestrol. This was found to be the case irrespective or whether the recoveries were calculated on the basis of wet or dry weight of hepatic tissue. No consistent differences were observed in the size of the livers of stilbestrol-treated and untreated birds. Hence it may be concluded that the whole

liver of the injected bird produces more phospholipids than the liver of the uninjected bird.

The results shown in Table 1 were obtained from livers that had been removed from birds at intervals of 6–16 hours after the injection of diethylstilbestrol. No increase in the rate of conversion of inorganic P^{32} to phospholipid P^{32} was observed in livers excised as early as 3 hours after the injection of the estrogen. Livers removed as late as 24 hours after a single injection of 10 mg. of diethylstilbestrol were also studied; increased phospholipid activities were observed at this later interval, but the results were more variable and the increases less pronounced than those recorded in Table 1.

Comment. Direct evidence that the liver is the main source of plasma phospholipids in the dog has been presented by Fishler, Enteman, Montgomery and Chaikoff (1943). They showed that only negligible amounts of radiophospholipids could be recovered from the plasma of hepatectomized dogs that had received radioactive inorganic phosphorus. An earlier observation of Hevesy and Hahn suggests that the liver could also be the site of formation of plasma phospholipids in the bird (1938). In laying birds injected with radioactive phosphorus, these workers observed that the specific activity of phospholipid phosphorus in the liver was higher than in plasma.

The increase in the level of plasma phospholipids of the bird produced by the injection of stilbestrol could result either from an increase in the rate of delivery of phospholipids by the liver to plasma or from a decrease in their rate of removal from plasma. The augmented rate of formation of radiophospholipid observed here in hepatic tissue excised from stilbestrol-treated birds suggests that this estrogen produces its effects by increasing the outpouring of phopholipids by the liver.

SUMMARY

The rates of incorporation of inorganic radiophosphorus into phospholipid by surviving slices of liver obtained from 1) normal birds and 2) birds that had received a single injection of 10–15 mg. of diethylstilbestrol were compared. A significant increase in the *in vitro* formation of radiophospholipid of the liver of the stilbestrol-treated bird was observed. This observation suggests that the increase in plasma phospholipids produced by the injection of diethystilbestrol results from an increase in the rate of formation of phospholipids by the liver.

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THE EFFECT OF THYROXIN ON THE RESPONSE OF THE THYROID GLAND TO THYROTROPIC HORMONE[1]

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PREVIOUS STUDIES on the effect of administered thyroid hormone on the thyroid gland have revealed the fundamental fact that when an animal receives an excess of exogenous thyroid substance, its own thyroid gland is put in a resting state, characterized by a decrease in total size, a diminution in height of thyroid epithelium and an accumulation of colloid (Herring, 1917; Cameron and Carmichael, 1920). Loeb (1929) further demonstrated that the compensatory hypertrophy of the thyroid gland resulting from partial ablation of the thyroid gland can be entirely prevented by administering thyroid hormone. Following the discovery of the existence of a thyrotropic hormone in the anterior pituitary gland, Aron (1930), Loeb, Bassett, and Friedman (1930) and Loeser (1934), showed that in guinea pigs the simultaneous administration of thyroid hormone along with an active extract of the anterior pituitary prevented in part the usual hypertrophy of the thyroid gland which follows the injection of thyrotropic hormone. Loeser (1934) postulated that this effect of thyroid hormone was the result of the suppression of the animal's own thyrotropic hormone secretion by the administered thyroid substance, leaving only the exogeneous thyrotropic hormone to stimulate the thyroid gland. He took as corroborating evidence the experiments of Kuschinsky (1933) and Hohlweg and Junkmann (1933) both of whom demonstrated a diminution in thyrotropic hormone content of the pituitaries of rats given thyroid hormone.

The investigations reported here were undertaken to determine whether the depressing effect of administered thyroid hormone on the action of exogenous thyrotropic hormone is entirely related to suppression of anterior pituitary thyrotropic hormone, or whether a similar effect can be obtained in the complete absence of the pituitary gland.

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METHODS

Three experiments were performed. In the first experiment immature female guinea pigs weighing between 170 and 200 grams were used. In the second experiment, which was carried out on hypophysectomized rats, Sprague-Dawley strain male rats weighing between 70–90 grams were used. In the last experiment, adult intact male rats of the Sherman strain, weighing between 100–150 grams were used. All the animals were kept on the stock laboratory diets throughout the course of the experiments.

The response of the thyroid gland to thyrotropic hormone was determined in all but one groups of rats (see below), by the histological appearance of the gland as well as by the measurement of the mean acinar cell height of the thyroid epithelium according to the method of Rawson and Starr (1938). The animals were sacrificed with ether, the thyroids removed and fixed in 10% neutral formalin. Paraffin sections were stained with hematoxylin and eosin. Each gland was examined under the oil immersion lens, and a representative cell in each of 100 consecutive acini was selected and its height measured. From these measurements the mean cell height of the thyroid epithelium was determined.

In one group of rats in the second experiment, the response of the thyroid gland was determined by the ability of the gland to take up a tracer dose of radioactive iodine.[4] Four hours before sacrificing the animals, each animal was injected intraperitoneally with 1 cc. of a solution containing 2 μg. of iodide as sodium iodide, labelled with a tracer amount of radioactive iodine, (half-life 8 days). Upon sacrificing the animals the thyroids were removed intact with the trachea and deposited in 3 cc. of 2% solution of NaOH for digestion. Aliquots of the NaOH digest were examined quantitatively for radioactivity, and compared with an equal aliquot of the originally injected solution. Thus the percentage of the administered radioactive iodine taken up by each animal's thyroid was determined.

Hypophysectomy was performed by the parapharyngeal route, using ether anesthesia. Completeness of operation during the course of the experiment was judged by the body weight curve, and was verified by careful examination of the pituitary region at the termination of the experiment.

The thyroxin[5] used in all the experiments was prepared in the following manner: crystalline thyroxin was dissolved in 0.1N NaOH, using 0.1 cc. for each mg. of thyroxin. This solution was then further diluted, depending upon the requirements of the experiment, to such a volume that each animal received either 0.25 or 0.5 cc. of solution at each injection. The injections were made subcutaneously. The thyrotropic[6] hormone was likewise given by subcutaneous injection. All hypophysectomized rats receiving thyroxin, along with their controls, were given 0.5 mg. of desoxycorticosterone acetate[5] daily by intramuscular injection. According to Schweizer, Ehrenberg and Gaunt (1943), desoxycorticosterone acetate improves the ability of hypophysec-

[4] We are indebted to Dr. Wendell Peacock of the Radioactivity Center of the Massachusetts Institute of Technology for supplying the radioactive iodine and for making the radioactivity determination.

[5] We are indebted to Hoffmann-LaRoche for generous supplies of crystalline thyroxin and desoxycorticosterone acetate (Doca) in oil.

[6] We are indebted to Parke, Davis & Company for a generous supply of Antuitrin-T.

tomized rats to tolerate thyroxin. This observation was confirmed in our experience.

RESULTS

The experiments of Aron (1930), Loeb, Bassett and Friedman (1931) and Loeser (1934), in which they demonstrated the depressing effect of thyroid hormone on the response of the thyroid gland of guinea pigs to thyrotropic hormone has been confirmed by us in a quantitative way by comparing the dosage-response curves of intact

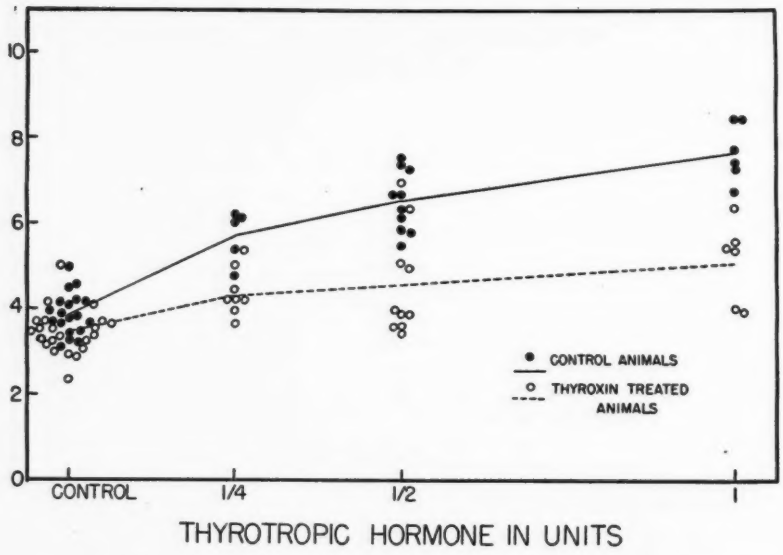

FIG. 1. The effect of thyroxin on the response of the guinea pig thyroid to thyrotropic hormone as indicated by the mean acinar cell height of the thyroid epithelium. Each point represents the mean cell height of a single animal's thyroid. 50 μg of thyroxin were given daily by subcutaneous injection for eleven days. Thyrotropic hormone was given daily for the last 3 days of the experiment.

immature female guinea pigs to thyrotropic hormone with and without treatment with thyroxin. Two groups of guinea pigs were used, one group of 46 animals receiving 50 μg. of thyroxin subcutaneously daily for eleven days, and a group of 40 control animals receiving a dilute solution of NaOH, the alkalinity of which corresponded to that of the solution containing thyroxin. On the 9th, 10th, and 11th days the various animals of each group received either 0, $\frac{1}{4}$, $\frac{1}{2}$ or 1 Junkman-Schoeller unit of thyrotropic hormone daily for the three days. The animals were sacrificed on the 12th day and their thyroids examined. The mean acinar cell heights of the thyroid epithelium of the individual animals are plotted in figure 1, and the means of all the values at each dosage level are recorded in table 1.

These results indicate that the continued administration of thyroxin will interfere with the normal stimulating effect of thyrotropic hormone on the guinea pig thyroid, as indicated by mean cell height determinations. The thyrotropic hormone output from the anterior pituitary gland of immature guinea pigs is quite low, as evidenced by the fact that the thyroid gland is in a resting state. Furthermore, assays of the animal's own pituitary show only slight thyrotropic hormone potency (Loeb, 1932). Nevertheless, the fact that the pituitary gland is present in these experiments on guinea pigs makes it impossible to determine whether the depressing effect of the thyroxin is a

TABLE 1. THE EFFECT OF THYROXIN ON THE RESPONSE OF THE GUINEA PIG THYROID TO THYROTROPIC HORMONE

Amount of thyrotropic hormone units	Mean acinar cell height of thyroid epithelium			
	Control		Thyroxin treated	
	No. of animals	Mean & S.E.	No. of animals	Mean & S.E.
0	19	3.86 ± 0.47	22	3.44 ± 0.52
$\frac{1}{4}$	5	5.70 ± 0.57	8	4.37 ± 0.52
$\frac{1}{2}$	10	6.51 ± 0.68	10	4.56 ± 1.16
1	6	7.67 ± 0.61	6	5.11 ± 0.88

direct effect upon the thyroid gland, or whether it is mediated through the hypophysis. Because of the ease with which the pituitary gland is removed in rats, an experiment similar to the one described above on guinea pigs was performed on a group of hypophysectomized Sprague-Dawley strain male rats.

The hypophysectomized rats were divided into two groups, consisting of 19 animals in each group. One group served as controls, the second were treated with 20 μg. of thryoxin daily for 10 days beginning approximately three weeks after operation. During this ten-day period all animals received 0.1 mg. of desoxycorticosterone acetate daily. Each group of animals was divided into three series, and on the 7th, 8th, 9th, and 10th days the animals in each series received 0, 4, and 8 Junkman-Schoeller units respectively of thyrotropic hormone daily for the four days. On the 11th day all 38 animals were sacrificed along with six unoperated, untreated control rats from the same group of animals. All of the thyroids were examined microscopically.

It should be noticed that much smaller doses of thyrotropic hormone were used in the experiment on the intact immature guinea pigs than were used here on the hypophysectomized rats. The amount of thyrotropic hormone that would give optimal increases in mean cell height per unit dose was previously determined for each animal, and those dosages were used in the present experiments. It is evident that the immature female guinea pig is more sensitive to thyrotropic hormone than the hypophysectomized rat.

Figure 2 shows the progress of the hypophysectomized rats during thyroxin treatment as indicated by the body weight curves. Those animals receiving thyroxin alone showed the same steady weight curve as the untreated hypophysectomized control rats. Those animals receiving thyrotropic hormone along with the thyroxin showed a slight decline in body weight as compared with animals receiving only

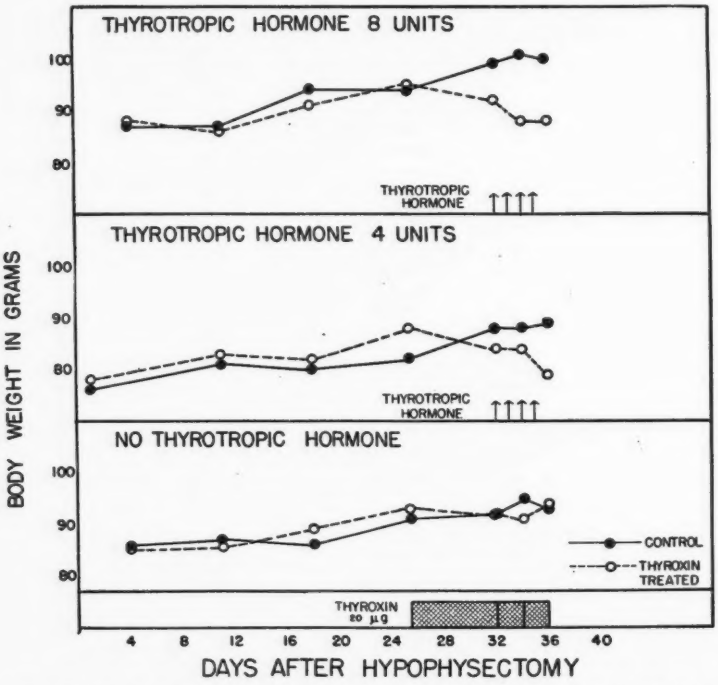

FIG. 2. The body weight curves of hypophysectomized rats treated with thyroxin alone and thyroxin plus thyrotropic hormone. Each curve represents the averages of all the animals in each group. The lower curve is for hypophysectomized rats receiving no thyrotropic hormone, the middle curve, for animals receiving 4 units of thyrotropic hormone, and the upper curve, 8 units of thyrotropic hormone. The thyroxin treated animals in each group were given 20 µg. daily for 10 days. The thyrotropic hormone was given daily for the last four days of the experiment.

thyrotropic hormone. In no case, however, was there any evidence of emaciation in any of the hypophysectomized rats.

Figure 3 shows the individual values of mean cell height of the thyroid epithelium for each hypophysectomized animal, plotted according to dosage of thyrotropic hormone. Table 2 contains the mean values for each group of rats at the different dose levels. Examination of the data reveals that the low thyroid epithelium resulting from hypophysectomy is not further depressed by treatment with thyroxin.

The injection of thyrotropic hormone in untreated hypophysectomized rats results in a definite stimulation of their thyroid glands, as measured quantitatively by mean cell height determinations, the degree of stimulation depending on the amount of thyrotropic hormone given. Although thyrotropic hormone likewise produces a stimulation of the thyroid gland of animals treated with thyroxin, the response is definitely less than in animals not receiving thyroxin. This

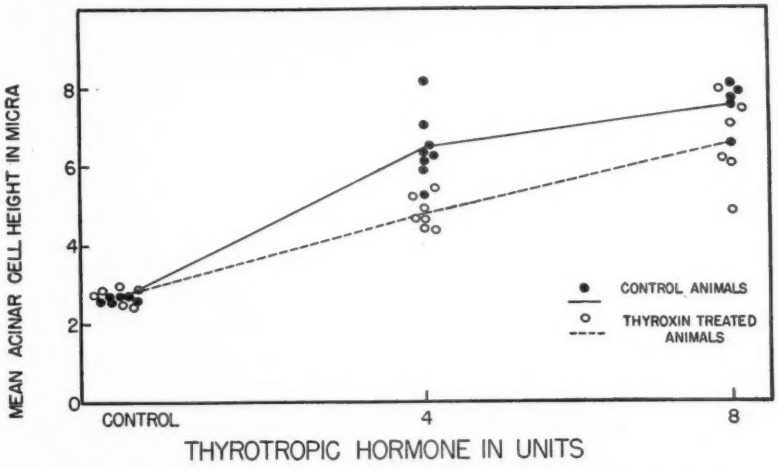

FIG. 3. The effect of thyroxin on the response of the hypophysectomized rat's thyroid to thyrotropic hormone as indicated by the mean acinar cell height of the thyroid epithelium. Each point represents the mean cell height of a single animal's thyroid. 20 μg. of thyroxin were given daily for ten days. Thyrotropic hormone was given daily for the last 4 days of the experiment.

depression of response is especially marked at the 4 unit dose level. With 8 units of thyrotropic hormone, the difference in response between the control and thyroxin treated animals is not as significant, probably because with this dose the range of maximal response to thyrotropic hormone has been reached. These results indicate that

TABLE 2. THE EFFECT OF THYROXIN ON THE RESPONSE OF THE HYPOPHYSECTOMIZED RAT TO THYROTROPIC HORMONE

Amount of thyrotropic hormone units	Mean acinar cell height of thyroid epithelium			
	Control		Thyroxin treated	
	No. of rats	Mean & S.E.	No. of rats	Mean & S.E.
0	6	2.64±0.069	6	2.73±0.19
4	8	6.47±0.80	7	4.81±0.37
8	5	7.53±0.53	6	6.57±0.93

in the absence of the pituitary gland, administered thyroid hormone can depress the response of the thyroid gland to exogenous thyrotropic hormone.

The ability of the thyroid gland to take up radioactive iodine can be used as an indication of thyroid gland activity under proper conditions. Leblond and Süe (1941) and Morton, Perlman, Anderson, and Chaikoff (1942) both demonstrated that removal of the hypophysis in the rat causes a marked diminution in the ability of the animal's thyroid to take up radio-active iodine. The thyroid glands of intact rats receiving exogenous thyroid hormone likewise show a markedy diminished uptake of radioactive iodine (Coleman, Litzenberger, Peacock and Rawson). In this experiment two groups of hypophysectomized rats were treated exactly as in the previous experiment, one receiving 20 μg. of thyroxin daily for 10 days, the other serving as controls. On the last four days of the experiment part of the animals in each group were given 4 units of thyrotropic hormone daily for the four days. All animals received a tracer dose of radioactive iodine 4 hours before death, along with six unoperated, untreated control rats from the same group of animals. Analyses of the thyroid glands for percentage uptake of radioactive iodine were carried out as described above. The results are presented graphically in Figure 4, each column representing one animal.

Unoperated, untreated control rats were found to take up an average of 6.98% of the administered dose of radioactive iodine, while hypophysectomized rats took up only 0.43%, an amount which was not further depressed by the administration of thyroxin. Four units of thyrotropic hormone given to hypophysectomized rats restored to the thyroid glands their normal ability to take up radioactive iodine (7.82%). However, when thyroxin was administered to hypophysectomized rats along with the thyrotropic hormone, the radioactive iodine uptake was found to be only 2.72%. These results again illustrate the ability of administered thyroid hormone to interfere with the response of the thyroid gland to thyrotropic hormone.

Because the thyroid glands were removed from the rats intact upon the trachea for radioactivity determinations, no thyroid weights were available, so that the figures given represent the total uptake for each gland, irrespective of size. However, the results from the previous experiment indicate an increase of approximately 2.5 times in cell height of thyroid epithelium following the administration of 4 units of thyrotropic hormone to a hypophysectomized rat. The present results indicate an increase of over 17 times in radioactive iodine uptake under similar circumstances. It would seem, therefore, that the changes in radioactive iodine uptake go beyond the changes due to differences in thyroid size alone.

The experiments reported above demonstrate that in the absence of the pituitary gland, thyroid hormone can suppress the response of

the thyroid gland to administered thyrotropic hormone. However, in the normal rat, suppression of the animal's own pituitary thyrotropic hormone secretion undoubtedly plays a role in the response of the animal's thyroid gland to exogenous thyroid hormone. It is known that excess thyroid hormone decreases appreciably the content of thyrotropic hormone in the pituitary glands of rats, and furthermore,

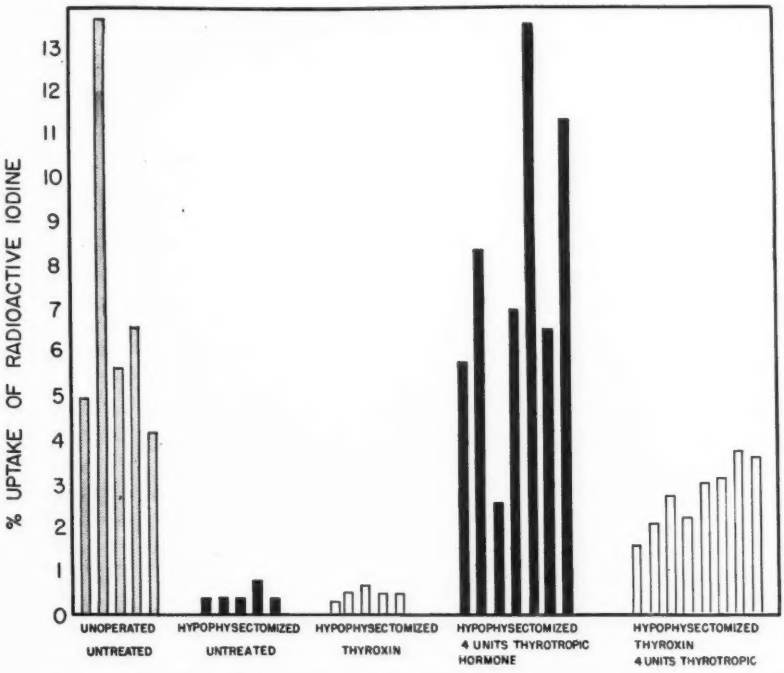

FIG. 4. The effect of thyroxin on the response of the hypophysectomized rat's thyroid to thyrotropic hormone as indicated by the % uptake of radioactive iodine by the rat's thyroid. Each column represents the % uptake of radioactive iodine by a single animal's thyroid gland. 20 μg. of thyroxin were given daily for ten days. Thyrotropic hormone was given daily for the last 4 days of the experiment.

injections of thyroxin produce changes in the histological appearance of the rat's pituitary (Severinghaus, Smelser, and Clark, 1934). The following experiment was performed to determine whether the suppression of endogenous thyrotropic hormone could be replaced by an excess of injected thyrotropic hormone.

Adult intact rats of the Sherman strain were used, and in order to ensure maximum suppression of the animal's own thyrotropic hormone, a large dose of thyroxin was administered for a relatively long time. In giving thyrotropic hormone a dose was chosen which would

be well above the amount necessary to maintain the normal cell height of the rat's thyroid. The animals were divided into two groups, one group receiving 0.1 mg. of thyroxin daily for 3 weeks, and the other serving as controls. During the last four days of the experiment the animals of each group were divided into three series, receiving 0, 20, and 50 units of thyrotropic hormone respectively daily for the four days. On the fifth day the animals were sacrificed, and the mean acinar cell heights of the thyroid epithelium of each animal were

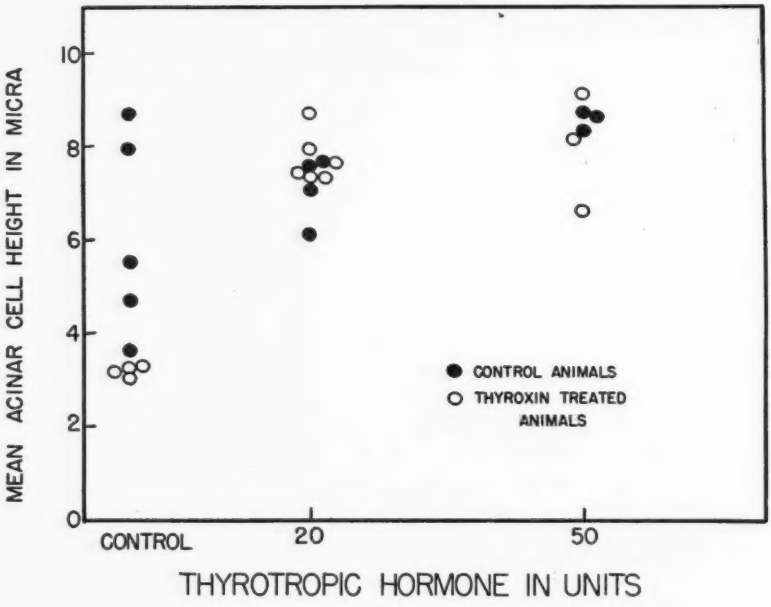

FIG. 5. The effect of thyroxin on the response of the adult intact rat's thyroid to thyrotropic hormone as indicated by the mean acinar cell height of the thyroid epithelium. Each point represents the mean cell height of a single animal's thyroid. 0.1 mg. thyroxin were given daily for three weeks. Thyrotropic hormone was given daily for the last 4 days of the experiment.

determined. These results are plotted in Figure 5, each dot representing one animal.

The variability in mean cell height shown here by the normal rats is not uncommon for adult intact rats. But treatment with thyroxin resulted in a depression of the thyroids to a constant level, averaging 3.20 micra. When thyrotropic hormone was administered to thyroxin treated rats, those animals receiving 20 units showed a mean cell height averaging 7.73 micra, while 50 units resulted in a mean cell height averaging 7.97 micra. The response of the normal rats to the thyrotropic hormone injections is somewhat difficult to estimate be-

cause of the variability among the control animals, but the data indicate that the relative response of these animals to thyrotropic hormone is definitely not as great as in the thyroxin treated animals.

DISCUSSION

The thyrotropic hormone secreted by the anterior pituitary gland is essential for the normal anatomy and physiology of the thyroid gland; the interrelationship between the two glands is mutual in that the secretion of the thyroid hormone likewise influences the pituitary gland, at least in respect to its thyrotropic hormone. It has generally been accepted that an excess of thyroid hormone suppresses the output of thyrotropic hormone from the pituitary, thereby resulting in a diminished activity of the thyroid gland itself. Whether thyroid hormone could inhibit the activity of the thyroid gland directly has been the cause for some speculation (Galli-Mainini, 1941). The experiments reported here on hypophysectomized rats, using mean cell height of the thyroid epithelium as well as uptake of radioactive iodine as criteria of thyroid activity, indicate quite clearly that the presence of circulating thyroxin definitely interferes with the response of the thyroid gland to injected thyrotropic hormone. The site of this action, is however, not apparent from these experiments. Thyroxin may have a direct effect upon the thyroid epithelium so that the cells cannot respond as well to the stimulus of thyrotropic hormone, or the circulating thyroxin may diminish the activity of the thyrotropic hormone before it even reaches the thyroid gland.

The anterior pituitary gland of the rat normally secretes a relatively large amount of thyrotropic hormone, so that the thyroid epithelium is usually in a rather high state of activity. The amount of exogenous thyrotropic hormone necessary to produce a given increase in cell height of the thyroid epithelium is great, compared, for example, to the amount of thyrotropic hormone necessary to produce a proportionate increase in the cell height of the thyroid epithelium of the immature guinea pig. This difference between the two animals is attributed to the fact that the rat's pituitary normally secretes a greater quantity of thyrotropic hormone and therefore the thyroid epithelium is already beyond its point of maximal response per unit dose of tropic hormone, at least in regard to changes in cell height. When thyroxin is administered to normal rats, part of its effect is to diminish the output of thyrotropic hormone from the anterior pituitary and thereby depress the thyroid gland to a lower level of activity.

The experiment reported here demonstrates that the height of the thyroid epithelium of an intact rat receiving thyroxin can be brought up to normal by the administration of a substantial dose of thyrotropic hormone. The injected thyrotropic hormone replaces the animal's own thyrotropic hormone, the secretion of which has been

suppressed by thyroxin. At the same time there is a sufficient excess of thyrotropic hormone to overcome the peripheral interference which thyroxin presents to the thyrotropic hormone response.

SUMMARY

The administration of thyroxin to the immature female guinea pig depresses the response of the animal's thyroid gland to exogenous thyrotropic hormone as determined by the mean cell height of the thyroid epithelium.

The administration of thyroxin to hypophysectomized male rats depresses the response of the animal's thyroid gland to exogenous thyrotropic hormone, as determined by the mean cell height of the thyroid epithelium, as well as by the ability of the thyroid glands to take up radioactive iodine.

The administration of thyroxin to adult intact male rats depresses the mean cell height of the thyroid epithelium. By the administration of an excess of thyrotropic hormone, the cell height can be restored to normal.

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FUNCTIONAL AND MORPHOLOGICAL ALTERATIONS OF THE REPRODUCTIVE SYSTEM OF THE FEMALE RAT FOLLOWING PREPUBERAL TREATMENT WITH ESTROGENS[1]

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WHEN LACTATING mother rats are treated with estrogens during the first 14 days of lactation, the young show an atypical pseudostratified uterine epithelium, cornified vaginal epithelium, and hyperplastic myometrium. Ovarian and Fallopian tube development are retarded (Weichert and Kerrigan, 1942). The present investigation is concerned with the after-effects of such premature stimulation in later life.

Greene and Burrill (1941) reported that female rats given single large doses of estradiol dipropionate at birth, when examined 3–18 months later, had markedly enlarged and inflamed oviducts, small ovaries lacking corpora lutea, and uteri showing squamous metaplasia. The animals remained in persistent estrus. Turner (1941) found that newborn female rats, given 100 to 200 I.U. of estrogen daily for the first ten days of life, later showed prolonged estrous periods, occasionally copulated without impregnation, and at ten months of age had ovaries containing only atretic follicles. Wilson (1943) observed that sexual behavior was permanently altered when young female rats were treated with small doses of estradiol dipropionate for 28-day periods. The most severe modifications resulted from treatment begun at birth, but treatment started on the 15th day or later had no permanent effect. At mature ages his animals showed a complete lack of spontaneous sexual behavior and small ovaries lacking corpora lutea but containing small atretic follicles and atrophic interstitial tissue. The uteri were small and showed several pathologic conditions.

The present report presents an account of the after-effects in female rats treated either with diethylstilbestrol or estrone in the

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manner described by Weichert and Kerrigan (1942). Also included are observations made on a large group of rats treated with diethylstilbestrol at early ages by other methods of administration.

MATERIALS AND METHODS

A total of 158 female albino rats of an inbred colony of the Sprague-Dawley strain was used. On the basis of methods of administration, they are grouped as follows: (1) Seven mothers of litters were given daily injections of 0.1 mg. diethylstilbestrol or 0.5 mg. estrone during the first 14 days of lactation. Ten females of the seven litters were placed with foster mothers at the end of treatment and reared to maturity. Ten additional females from 3 other litters were treated similarly from day 7. (2) Eighteen females from 9 litters were given 1 to 14 daily injections of 0.01 mg. diethylstilbestrol beginning on the 7th, 14th, 21st, or 28th days. Since the vaginae of 61 untreated littermates opened prematurely merely as a result of their being caged with the injected animals, they were retained for study along with 59 other animals whose vaginae had opened prematurely. The latter animals had been in cages in which the nesting material had been sprayed with a solution of diethylstilbestrol and ether (0.1 mg. diethylstilbestrol in 20 cc. of ether) when the animals were either 2 or 8 days old. In some cases the cages had been treated only once; others had been sprayed as many as 5 times in a period of 10 days. The cages had purposely not been cleaned for 21 days or longer.

Daily vaginal smears were taken from the time that the vaginae opened. At maturity many animals were caged with vigorous males whose fertility was afterward tested with normal females. A few attempts were made to determine the reactions in the adult state to estrogens and to a gonadotrophic preparation.[2] Unilateral ovariectomy was performed in several cases in an effort to alter vaginal sequences. Material for histological study was taken from representatives at ages ranging between 14 and 460 days. Twenty-one normal animals were used as controls.

RESULTS

Time of vaginal patency. The vaginae of the young of estrone-treated mothers opened after 9–10 days, whereas those of stilbestrol-treated mothers did not show this response for 10–13 days. Vaginae opened in 10–13 days in those given direct injections of stilbestrol and those merely caged with injected littermates. In females in contact with stilbestrol-contaminated shavings, the time of vaginal patency was 19–20 days after a single spraying and 11 days after 5 sprayings. The average age for the establishment of the vaginal introitus in 21 normal control females was 49 days.

Estrous cycles. The ages at which estrous cycles first appeared in the experimental animals were similar to the ages at which puberty occurred in the control animals. Cycles were either aberrant from the very beginning or became so after 4 to 10 months. Some animals

[2] Antuitrin-S, Parke, Davis and Co., Detroit, Michigan.

had recurring periods of prolonged estrus, usually without a proestrous stage, but many remained in vaginal estrus for extended periods. One outstanding case was that of an animal for which no diestrous smears were recorded during the entire life span of 381 days. Only 2 cases out of 84 were of the anestrous type; but even in these there were prolonged estrous intervals on rare occasions. Animals treated at earlier ages were the more greatly affected (Table 1).

Vaginal smears often contained thick masses of cornified cells and large numbers of leucocytes. This type of smear was recorded as metestrus, but histological studies showed cornified vaginae. The pres-

TABLE 1. TIME OF TREATMENT WITH ESTROGENS IN RELATION TO ESTROUS BEHAVIOR

Age when given initial treatment	Type of estrous behavior		
	Cyclic	Persistently estrous	Anestrous
(days)	(no. animals)	(no. animals)	(no. animals)
1–2	4*	55	2
7–8	4	3	0
13–14	3	3	0
21–22	7	0	0
28	3	0	0

* Received lowest dosage; vaginae opened 19–20 days after beginning of treatment.

ence of leucocytes was due to inflammations in the upper part of the reproductive tract.

Smear records for 21 control animals showed no tendency toward persistent estrus, although smears were taken daily until these animals were a year old.

Mating behavior and reproduction. Although the majority of animals treated before the 15th day copulated when tested at mature ages, only one out of 36 became pregnant and delivered young. This animal had been in contact with shavings sprayed only once with diethylstilbestrol. Cycles were normal both before and after this gestation, but the animal failed to become pregnant again.

A few cases of pseudopregnancy were noted. All members of the group treated for the first time at 21 days of age had normal cycles, copulated, and bore litters. Two later failed to become pregnant, and autopsies showed bilateral pyosalpinx. Two others exhibited masculine copulatory behavior, but it was observed on only one occasion in each case.

Reactions to hormonal preparations. Anestrous animals, when treated for 15 days with diethylstilbestrol (either 0.01 mg. or 0.1 mg. per day), exhibited fully cornified smears throughout the test period. Animals with a tendency toward persistent estrus, when treated during a diestrous period, showed the same reaction. In contrast,

normal control animals of the same age exhibited estrus only during the first part of a similar test period. Daily injections of 100 R.U. of (chorionic) gonadotrophin[2] into a constant-estrous animal produced diestrous smears in 3 days. After 10 days of treatment, histological preparations showed a mucified vaginal epithelium.

Effect of unilateral ovariectomy in persistent-estrous animals. Two animals were unilaterally ovariectomized when in estrus, and diestrous smears appeared on the following day. Cornified smears reappeared in one of these animals but after 30 days. Unilaterally ovariectomized control animals exhibited normal vaginal sequences throughout the post-operative period.

TABLE 2. OCCURRENCE OF TUBAL INFLAMMATIONS AFTER ESTROGEN ADMINISTRATION

Age when given initial treatment	Method of administration	Animals with inflammation	Unaffected animals
(days)			
1–2	from lactating ♀	9	0
	from littermates	2	3
	from shavings	39	12
7–8	from lactating ♀	2	1
	injected	2	0
	from littermates	3	0
	from shavings	0	8
11–14	injected	1	2
	from littermates	3	14
21–22	injected	1	8
	from littermates	1	17
28	injected	0	2
	from littermates	0	14
Colony controls	untreated	20	779

Morphological changes. Out of a group of 15 animals given 14 days' treatment starting at ages earlier than 14 days, 10 showed abnormal conditions in the periovarial bursae or oviducts at the end of treatment. Ovaries were slightly retarded, compressed and displaced. The bursae were either distended with blood, with blood and polymorphonuclear leucocytes, or abscessed. In the latter condition, the Fallopian tubes were also involved. Conditions in the uteri, cervices, and vaginae indicated a high estrogenic stimulation but no inflammation.

The pathological findings in 71 animals treated for the first time within the first two weeks of life and reared to adulthood included pyometra, endometritis, cystic glandular hyperplasia, squamous metaplasia of the epithelium of both uterus and Fallopian tube, pyosalpinx, and chronic salpingitis. The incidence of pyosalpinx and chronic salpingitis in relation to age of treatment is given in table 2.

Inflammatory conditions in the oviducts varied from only a moderate swelling of the cephalic part of the tube to large tubo-ovarian cysts. Adhesions frequently connected these cysts to other viscera or to the body wall. One of the largest weighed slightly more than 13 gm. and measured 48 mm. through its longest diameter. In one animal a necrotic condition appeared over the site of the abscess causing the hair to fall out and the skin to blacken and later ulcerate. In one animal, when a large cyst became palpable, it and the uterine horn from the same side were removed under aseptic conditions and placed in separate tubes of nutrient broth. B. pyocyaneus was obtained in pure culture from both.

Ovaries from 38 animals of various ages ranging between 42 and 460 days were studied histologically. In general, they contained a few developing follicles, wide areas of interstitial tissue, and large vesicular follicles. Corpora lutea were found only in those in which cycles had become aberrant after 4–10 months of age, and these were old and degenerate. The longest diameter of the vessicular follicles usually measured no more than 0.9 mm., but three cases stand out because of their extreme sizes, which were 16.8 mm., 5.4 mm., and 3.7 mm.

Only 2 animals with abnormalities were found among 43 treated for the first time at 21 days of age or later. Cycles had been normal in both cases, and the ovaries were normal except for an enormous cystic follicle in one animal. Both animals had pyosalpinx, and one had had one pregnancy.

It was possible to establish a figure for the natural incidence of tubal inflammations in this colony of rats since a large number of preserved specimens of reproductive tracts from mature females was available. Only 20 cases were found in 799 animals. None of these had the character of a tubo-ovarian cyst. All of the 799 animals had been smeared over extended periods and many as long as the epxerimental animals and at the same time. The same instruments were used throughout.

DISCUSSION

The results obtained in this study indicate that early prepuberal treatment with diethylstilbestrol or estrone for short periods and with fairly low dosages produces certain lasting effects in the female rat which have been reported when other estrogenic preparations were used (Greene and Burrill, 1941; Turner, 1941). Whereas the results in these experiments vary from those reported by Wilson (1943), who used estradiol dipropionate, it may be that they differ only in degree. Prepuberal treatment started prior to the 15th day in both sets of experiments altered sexual behavior. In his animals, cycles were completely abolished, and the ovaries contained only small and

atretic follicles and atrophic interstitial tissue; in the present experiments, persistent-estrus resulted, but the ovaries contained large cystic follicles and the interstitial tissue was not generally atrophic. Wilson concluded that prepuberal treatment with estrogen had so altered pituitary function that luteinizing hormone was not secreted. It is generally agreed among investigators who have concerned themselves with the problem of the persistent-estrous animal (Everett, 1939; Pfeiffer, 1939; Turner, 1941a and b; Bradbury, 1941; Greene and Burrill, 1941; Fels, 1941 and 1942; and Pfeiffer, 1942) that this phenomenon is the result of lowered LH secretion. It therefore seems that the effect noted by Wilson (1943) is more severe but not different.

The response shown by adult animals in the present study during treatment with estrogens is unlike that of normal adults but is similar to that of castrated animals (Morrell and Hart, 1941). The reaction to (chorionic) gonadotrophin is in agreement with tests of Greene and Burrill (1941) and Pfeiffer (1942) on constant estrous rats. The fact that unilateral ovariectomy brought on diestrus indicates that the amount of estrogen secreted in the persistent-estrous animal is relatively small.

The high incidence of bursal and tubal inflammation in both young and mature animals is perhaps the most significant feature of the results. Such conditions were not found by Wilson (1943). Inflamed oviducts have been found after long-continued treatment with gonadotrophins, androgens, and estrogens, after treatment with large doses of estrogens, and in rats in which persistent-estrus had appeared spontaneously (Everett, 1939; Fluhmann, 1939; Shay, Gershon-Cohen, Paschkis and Fels, 1939; Wilson, Young and Hamilton, 1940; Wilson, Hamilton and Young, 1941; Bradbury, 1941; Greene and Burrill, 1941; Turner, 1941a and b; Fels, 1941 and 1942; Greene, Burrill and Ivy, 1942; and Lacqueur, 1944). In the present study only low dosages and short periods of treatment were used, yet the conditions were as extreme as with long-continued treatment. If a speculation may be permitted, it would seem that the treatment of infantile rats with estrogens causes periovarial hemorrhage which, in turn, leads to inflammatory conditions in the bursa and secondarily in the oviduct. Since estrogens induce premature vaginal patency, it is easy for bacteria to gain access to the reproductive tract. The inflammatory lesions remain during development and are seen in the adults as enormously enlarged tubal abscesses. The smearing technique does not seem to be an important factor since only 20 cases of pyosalpinx appeared among 799 animals smeared for extended periods. These were less severe and not associated with abnormal ovaries or bursae. The instruments used were the same as were used for vaginal smears in the experimental groups. Out of 101 animals given treatment on the 14th day or earlier, 61 had severe tubal inflammation.

It seems significant that these effects may be induced in animals unintentionally administered small amounts of diethylstilbestrol at early ages. In fact, the results among such cases were more extreme than in animals treated directly.

These results may have clinical application since estrogens are employed in the treatment of vulvo-vaginitis in children.

SUMMARY

The administration of diethylstilbestrol or estrone to female rats beginning either at birth or as late as the 14th day and for so short a time as 14 days produced permanent functional and morphological changes in the reproductive system. Typically, these animals tended to remain in vaginal estrus. Cycles were usually aberrant from the time of puberty, but in a few cases persistent estrus did not appear for 4 to 10 months. Persistent-estrous animals copulated repeatedly, but none became pregnant; whereas animals treated for the first time at 21 days had normal cycles, copulated, and bore litters. Vaginal estrus was induced readily and could be maintained when treated animals in diestrus were injected with diethylstilbestrol or estrone. Diestrous smears were induced in constant estrous animals by the administration of (chorionic) gonadotrophin and by unilateral ovariectomy. Ovaries of adult animals given prepuberal treatment were generally small and contained a few developing follicles, numerous large vesicular follicles, and a considerable amount of interstitial tissue. A high incidence of bursal inflammation, pyometra, cystic glandular hyperplasia, and squamous metaplasia in the uterus and Fallopian tube was noted. Pyosalpinx or chronic salpingitis occurred in 61 per cent of the cases initially treated on or before the 14th day of life. Large tubo-ovarian cysts were found frequently. One of these, and a portion of the uterus, removed aseptically, showed B. pyocyaneus in pure culture.

It is significant that these conditions appeared in many animals treated accidentally by being in contact with other rats injected with diethylstilbestrol or in cages contaminated with this preparation.

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EVIDENCE SUGGESTING A RÔLE OF THE LACTOGENIC HORMONE IN THE ESTROUS CYCLE OF THE ALBINO RAT

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RECENT experimental studies of "persistent-estrous" rats (Everett, 1940, a, b; 1943) have shown that progesterone induces these animals to experience essentially normal estrous cycles. The evidence presented (1943) indicated that in bringing about corpus luteum formation this agent was most effective if its administration was timed to coincide with proestrus. This finding was regarded as indirect confirmation of evidence from other sources (Dempsey, *et al.*, 1936; Astwood, 1939; Boling and Blandau, 1939; Boling, Barton and Burr, 1940) that progesterone is secreted in the normal animal for a short time before ovulation takes place. The view was advanced that in the persistent-estrous rat it fails to be secreted and that in the normal rat during proestrus the corpus luteum hormone serves as a safety factor tending to assist, but not necessarily essential for, ovulation. It was suggested that at certain stages of the pituitary cycle in the rat, progesterone facilitates the release of luteinizing hormone.

The action of progesterone as a "safety factor" was observed in rats subjected to continuous light. Continuous illumination readily induced prolonged estrus (Browman, 1937; Hemmingsen and Krarup, 1937; Dempsey and Searles, 1943) in young cyclic rats of our DA strain (Everett, 1940, a; 1942). However, this effect was prevented by daily administration of small quantities of progesterone (1940, a), the animals remaining cyclic during the course of progesterone treatment. Subsequently, comparative studies were made of the response to continuous light by the DA and Vanderbilt strains,[1] respectively (Everett, 1942). Young Vanderbilt animals failed to show any significant prolongation of estrus, remaining cyclic through more than 30 days of continuous light, while DA animals of similar age began to show prolonged estrus within 10 days. Such a difference could be explained by the assumption that the Vanderbilt rats were protected by the secretion of a certain amount of progesterone, so regulated in quantity and timing that estrus and ovulation were not suppressed.

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[1] There is every reason to believe that the Vanderbilt strain, a derivative of the Osborne-Mendel strain, is "normal" stock with respect to the reproductive mechanisms.

These two strains (Vanderbilt and DA) have been maintained together in the same room under identical conditions for four years. Certain differences between them, other than indicated above, are pertinent with respect to the present report. The incidence of spontaneous "pseudopregnancy" is negligible in the DA strain. The few cases of greatly prolonged diestrus have accompanied acute illness.[2] The Vanderbilt animals on the other hand, have frequently experienced periods of prolonged diestrus during which the corpora lutea became markedly enlarged and resembled histologically those of a standard pseudopregnancy. Furthermore, as shown below, corpus luteum cells of *cyclic* Vanderbilt rats are significantly larger than those of DA rats at comparable stages. Considerable difficulty has been encountered in breeding the DA strain partly because of repeated irregularities of gestation in some animals, but chiefly because many of them fail to lactate adequately after an apparently normal pregnancy and the delivery of a large and seemingly healthy litter. The Vanderbilt stock has rarely shown any irregularity of gestation. Ninety per cent of its litters have been successfully nursed to weaning.

The outstanding differences with which we shall be concerned at present, then, are these: *a*) The Vanderbilt rats lactate much better than DA rats. *b*) They frequently experience spontaneous pseudopregnancy while DA rats do not. *c*) The corpus luteum cells of cyclic Vanderbilt rats are larger than those of DA rats. *d*) When young, the Vanderbilt animals are naturally "protected" against the action of continuous light in a manner reminiscent of the protection given DA rats by injected progesterone. A suggestion that these several differences have a common basis may be obtained from recent studies of the "luteotrophic" action of the lactogenic hormone (Astwood, 1941; Evans, Simpson, Lyons and Turpeinen, 1941). A subnormal "potential" for lactogen secretion is implied in the DA stock. Eventually, it is to be hoped, direct tests of this question may be made. Meanwhile, it was necessary to determine whether lactogenic hormone can cause secretion of progesterone at sufficiently low levels that regular cyclic ovulation will not be suppressed, yet adequate to restore cycles to persistent-estrous rats. Evidence to this effect has been obtained from the experiments reported below.

In pursuing this question certain conditions had to be met. The length of an induced cycle should be similar to the normal, without any considerable delay in the recurrence of estrus. The corpora lutea should resemble those of the cyclic Vanderbilt rat at comparable stages. Thus, an histological study of the corpora lutea was a necessary control for the adequacy of any particular lactogen regime. It was also necessary to consider Astwood's evidence (1939) which would seem to indicate that, if progesterone is formed at all during the nor-

[2] This is exclusive of the "short-day anestrus" which was often seen in DA animals before standard lighting conditions were established in the colony (Everett, 1942).

mal rat cycle, it appears principally during proestrus. Some degree of fluctuation might thus be postulated in the "luteotrophic" stimulus (Astwood, 1941; Hisaw and Astwood, 1942).

MATERIALS AND METHODS

Most of the experiments were designed as follows: Certain DA animals, selected as in previous investigations (Everett, 1940, a, b; 1943) for long histories of persistent estrus, were injected with 1.0 mg. of progesterone to interrupt this condition. A subsequent injection of 0.5 mg. of this substance was given, usually during proestrus, with the intention of inducing a cycle and a set of corpora lutea. As in the previous studies, several animals, although completing a vaginal cycle under these conditions, failed to luteinize. This circumstance produced one group with luteal tissue and another group without it. In both instances daily injections of lactogen[3] were instituted within 24 hours after the last progesterone treatment, usually when the vaginal smear showed full cornification.

The optimum doses of lactogen for this purpose were estimated from the amounts used by others to maintain rat corpora lutea in full activity (Evans, *et al.*, 1941; Tobin, 1942). Tobin found that Schering prolactin maintained strongly active corpora lutea when injected at a level of 17 I.U. or more each day. This is approximately the minimum daily dose which consistently maintained active corpora lutea in the experience of Evans, *et al.* Such a stimulus probably causes the secretion of the equivalent of 1.5 to 2.0 mgs. of injected progesterone per day. For the present experiments, progesterone secretion at a lower level (corresponding to 0.5 to 1.0 mg. injected daily) was desirable.

The lactogen treatments varied in different experiments. These will be presented in three series. In series I most of the procedures were comparable in that lactogen injections began with 5 I.U. doses. In one group this dose level was maintained through the subsequent proestrus and the first day of full cornification. In other cases the daily injections were increased to 15 I.U. in late diestrus or proestrus. Series II includes experiments in which it is believed that strong luteotrophic stimulation continued throughout the period of lactogen treatment, the animals entering proestrus well after the last lactogen injection. Here the doses were as large as 30 I.U. per day. Series III comprises experiments in which fluctuating dosages (4 to 30 I.U.) were employed, with attempts to produce at least two cycles in sequence.

Three different samples of purified sheep prolactin were used. Two of these were furnished in the form of dry films in ampoules marked 2PS1 and 2PS3, respectively. The solvent was furnished separately by the manufacturer. The other sample, a powder, was designated 46-51 pr 4 and was dissolved in distilled water as needed. All the solutions were prepared in a concentration of 100 I.U. per cc., based on the manufacturer's assay. With respect to the possibility of luteinizing activity in this material, it is believed that the 16 experiments, in which lactogen was administered in the absence of lutein tissue, adequately rule this out as far as the dose levels employed in this investigation are concerned. The administration of these amounts of

[3] I am indebted to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N. J., for a generous supply of progesterone and prolactin.

lactogen did not produce luteinization of mature follicles in ovaries which contained no corpora lutea. Apparently the lactogen contained either no luteinizing agent or only ineffectual traces. It is conceivable that in these cases the failure of ovulation during the progesterone cycle automatically selected the least responsive individuals, but such was not our experience in successive cycles induced by progesterone (Everett, 1943). For example, in 8 such experiments in which the first "progesterone" cycle was anovulatory, the next induced cycle was accompanied by corpus luteum formation in 7 instances.

Vaginal smears were made routinely each morning by means of saline lavage. The ovaries of the various animals were usually observed and removed at autopsy on the first day of diestrus after the completion of a vaginal cycle or, in case vaginal cornification persisted, the observations were made about 48 hours after a proestrous smear was obtained. The ovaries, with fallopian tubes attached, were routinely fixed in Zenker's fluid. At least one ovary from each animal was serially sectioned at 8μ, and every 12th section was mounted and stained by a modified Mallory connective tissue stain (Everett 1940, b; 1943).

Measurements of cross-sectional areas of corpus luteum cells were made in certain cases for comparison with similar cells in cyclic rats of the Vanderbilt and DA strains. The plan was to select at random 3 sections from each of 3 corpora lutea in each animal, these corpora having formed during the preceding cycle. At 1000 diameters magnification, camera lucida tracings were made of 50 cells selected at random from each corpus luteum. The areas of the various tracings were measured with a planimeter, the average area was determined for each animal and this value was corrected to represent the actual average cross-sectional area in square microns. The averages obtained for the several rats in each group were then treated statistically as individual determinations. The usual formulae for small numbers were applied to estimate the significance of differences among the various means.

One source of experimental error in the progesterone studies also applies to the prolactin experiments, e.g. the possibility of spontaneous occurrence of cycles in spite of treatment rather than because of it. This error has been estimated at 5 to 10 per cent (Everett, 1943).

RESULTS

In preliminary experiments it appeared that, in the absence of luteal tissue, the administration of lactogen had none of the effects which are produced in such animals by progesterone. However, if corpora lutea were first induced to form by progesterone treatment, a small quantity of lactogen given daily during the subsequent diestrus and early estrus resulted in the completion of a new ovulatory cycle. In further study of the problem these observations held true.

Series I. Among the 22 rats in this series, the initial "progesterone" cycle was accompanied by corpus luteum formation in 14 cases. The remaining 8 rats did not form corpora lutea and, hence, were devoid of lutein tissue during subsequent lactogen treatment.

Of the 14 rats with corpora lutea, 8 were given 5 I.U. of lactogen (sample 2PS3) daily throughout the following diestrus, proestrus and the first day of renewed full cornification (fig. 1, A). This resulted in a consistent prolongation of diestrus as illustrated. Seven of the animals were successfully carried through at least one "lactogen" cycle and the formation of new corpora lutea was confirmed histologically in each case. In one of these rats the experiment was continued through an additional cycle. The second diestrus was not prolonged. At autopsy

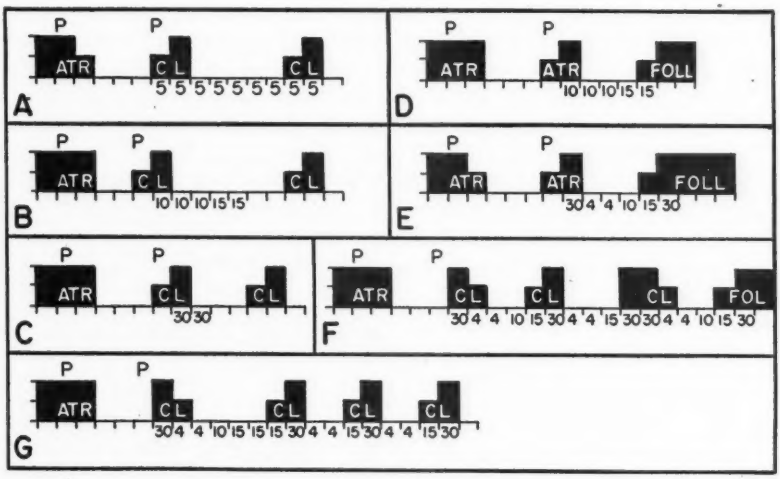


FIG. 1. Diagrams illustrating typical experiments. The solid black areas represent degrees of vaginal estrus. One division of the ordinate: proestrus or metestrus; two divisions: full cornification. The base line itself indicates diestrus. Each division of the abscissa represents one day. P: an injection of progesterone, as indicated in the text. Doses of lactogen (in international units) are shown by the numerals below the base lines. ATR: atresia of large follicles. FOLL: persistence of large follicles. CL: corpus luteum formation.

corpora lutea were found, corresponding to each observed cyclic estrus. In the eighth rat persistent estrus reappeared in spite of lactogen treatment.

Four animals possessing corpora lutea received daily injections of 5 I.U. of lactogen (sample 2PS1) during early diestrus. The dose was increased to 15 I.U. either on the third day of diestrus or during proestrus. Diestrus was not prolonged in these 4 cases. In one rat of the 4, persistent estrus reappeared in spite of treatment. Another failed to ovulate, but lutein cysts containing a considerable mass of new luteal tissue were found. In the other two, tubal ova and healthy new corpora lutea were observed in histological sections after the completion of the lactogen cycles.

Two remaining experiments are to be considered with the above. Both animals received lactogen (sample 2PS3) at a 10 I.U. level during early diestrus. In one, this level was continued through proestrus; in the other, it was increased to 15 I.U. in late diestrus. Diestrus was not prolonged in either instance and both animals completed a cycle with formation of new corpora lutea.

The 8 rats which did not form corpora lutea during the "progesterone" cycle were treated as follows: 5 I.U. of lactogen (2PS1: 2 rats; 2PS3: 2 rats) were administered daily throughout diestrus and the first 2 days of vaginal estrus. Two additional animals received 5 I.U. per day (2PS1) during early diestrus and 2 others (fig. 1, D) were started at a 10 I.U. level (2PS3). The dose was increased in these 4 cases to 15 I.U. in late diestrus and proestrus. In all of the 8 experiments persistent estrus reappeared. Diestrus was not significantly prolonged in any instance.

Series II. In this series of 14 animals an initial set of corpora lutea had been formed in every case through the mediation of progesterone. The definitive series comprises 9 experiments in which the corpora lutea were strongly stimulated by lactogen treatment confined to stage III of the progesterone cycle and the first part of the following diestrus.

Of the 9 animals, 3 were treated like the case illustrated in figure 1, B: 10 I.U. of lactogen during stage III of the progesterone cycle and on the first 2 days of diestrus, the dose increasing to 15 I.U. on the third and fourth days. Another rat received 5 I.U. daily through the second day of diestrus and 30 I.U. daily for the next 3 days. The remaining 5 animals were given an initial dose of 30 I.U. which was continued through the second day of diestrus in one instance and through the first day in the others (fig. 1, C).

In all of these 9 experiments proestrus invariably reappeared 3 days after the last injection. The duration of diestrus, therefore, appeared to be directly determined by the duration of lactogen treatment. The completion of ovulatory cycles was demonstrated in every case, save one of the 4 animals which received 30 I.U. for only 2 days. In this one animal the new estrus persisted. The other 3 rats which were treated in this same manner produced vaginal smear sequences which were entirely comparable with that in the normal 5-day cycle.

This fact raised the question whether a strong stimulus, confined to stage III of the progesterone cycle, would be similarly effective. In answer to this, indirectly, are the results of 5 experiments which were performed as a preliminary to series III. An injection of 30 I.U. was given in each of these during full cornification in the progesterone cycle. It so happens that this treatment was followed by 2 I.U. per day during diestrus. In 2 rats this dosage was continued into the following estrus. In the other 3 experiments the dose was even increased after 3 days to 15 I.U. and then 30 I.U. as the animals came into "heat."

Only 1 rat (in this latter group) completed an ovulatory cycle, persistent estrus reappearing in the others. The successful completion of the one cycle is probably to be ascribed to the full sequence of injections rather than simply to the 30 I.U. administered at the beginning.

Series III. Since the daily injection of 5 I.U. of lactogen 2PS3 had so consistently prolonged diestrus whereas 2 I.U. appeared inadequate, the following sequence of injections was decided upon: 30 I.U. in stage III of the "progesterone" cycle, 4 I.U. as a maintenance dose during early diestrus, followed by increasing amounts in late diestrus and proestrus. Six animals, possessing corpora lutea induced by progesterone, were so treated, but 2 returned to persistent estrus. The other 4, however, were all carried through at least 2 successive lactogen cycles (fig. 1, F), 1 completing a third (fig. 1, G). Sections of the ovaries contained corpora lutea representative of the observed cycles. In 2 cases it was not possible to demonstrate ovulation in the last cycle because of the timing of autopsy, but tubal ova were found in the other 2, thus proving ovulation in the final cycles of those animals.

Eight experiments were obtained by the above lactogen regime (sample 2PS3) in animals which had failed to ovulate and luteinize during the progesterone cycle (fig. 1, E). All of these returned to persistent estrus as in the illustrated example.

This series was amplified by the addition of 6 experiments in which a new sample of lactogen (46-51 pr 4) was employed. All 6 of these animals luteinized during the "progesterone" cycle. The lactogen regime differed from the above in that 5 I.U. were given on the first 2 days of diestrus, and increased to 15 I.U. uniformly on the following day. Of the 6 rats started, 2 returned to persistent estrus, while 4 experienced at least 1 "lactogen" cycle with new corpus luteum formation. Among the latter, 2 animals were successfully carried through an additional cycle, 1 was killed in proestrus of her second "lactogen" cycle and the remaining animal was apparently on the way to persistent estrus at the time of autopsy, for her follicles were still unruptured on the second day of full cornification, in spite of continued treatment.

Compilation of all the attempts in series III to induce cycles by lactogen in the presence of an initial set of corpora lutea, shows that among 22 potential cycles 16 (73%) were successfully completed with formation of new corpora lutea. This proportion is very similar to the degree of success obtained when injected progesterone was the agent (Everett, 1943).

Histology of corpora lutea. It was noted above that certain histological differences exist between the ovaries of cyclic DA and cyclic Vanderbilt rats, particularly with reference to the corpora lutea. Most of the observations presented here will refer specifically to the next youngest set of corpora lutea in ovaries removed on the first day of diestrus.

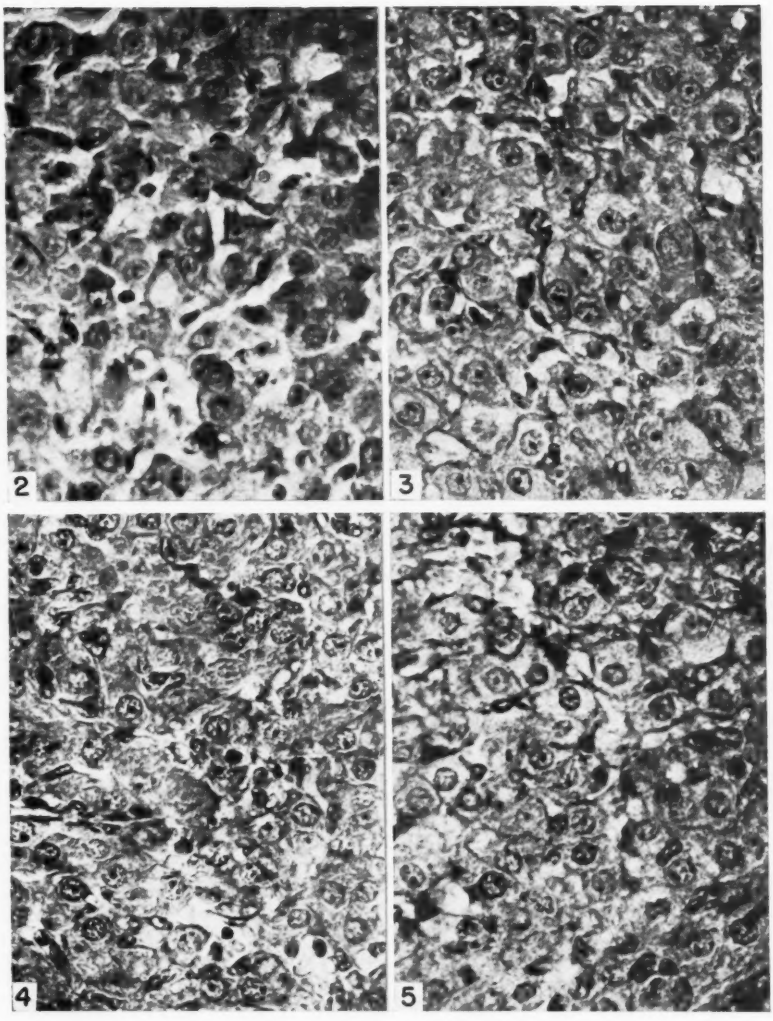
The luteal cells of cyclic DA animals (fig. 2) are smaller than those of Vanderbilt rats (as demonstrated below) and stain more intensely as a result of a greater density of the cytoplasm. Only occasionally do a few cells with extensively vacuolated cytoplasm occur. Cell outlines are difficult to discern. These features are in strong contrast to the characteristics of Vanderbilt corpora lutea (fig. 3), where great numbers of the cells are vacuolated and cell outlines are sharply delineated. The order of difference is notably similar to that which Corner (1942) noted in the macaque ovary, between corpora aberrantia and standard corpora lutea in retrogression.

The corpora lutea of persistent-estrous DA rats which were rendered cyclic by progesterone treatment alone (Everett, 1943) are similar to those of naturally cyclic DA rats (fig. 4). Vacuolated cells rarely occur in these cases. However, corpora lutea which were induced in like manner, but which were subsequently acted upon by lactogen in the experiments outlined above, were found to be significantly changed.

In series I the corpora lutea closely approached the appearance of those in the normal (Vanderbilt) rat. The resemblance was most complete when the diestrous interval had been of normal duration. When it had been prolonged, the cytoplasm of the luteal cells tended to be more densely stained and extensive patches of vacuolated cells were rarely present. In series II, the older corpora lutea were not impressively similar to the normal cyclic corpus luteum, save in compactness of the parenchyma. Groups of vacuolated cells occurred most frequently in the cases in which lactogen treatment had continued for several days (fig. 1, B). These corpora lutea were, however, somewhat larger than the normal. In the experiments of series III, histologic replicas of Vanderbilt corpora lutea were regularly found (fig. 5). This was true even in the few instances in which new ovulation failed in spite of the existence of older lutein tissue, a fact which supports an interpretation that these failures occurred in spite of the secretion of progesterone.

The vacuolation of cells in Vanderbilt corpora lutea (and in the replicas produced in the lactogen experiments) represents sudanophile inclusions,[4] often in the form of coarse droplets. The cells containing the greatest amount of this material tend to be concentrated in large, irregular patches in the interior of the corpus luteum. These patches closely resemble the patches of vacuolated cells illustrated by Boling (1942) in corpora lutea of similar age, but are often more extensive than illustrated in his paper. Ordinarily, DA corpora lutea are relatively free of sudanophile material, especially after proestrus. A more detailed study of this matter will be reported elsewhere, where it will be shown that by means of the lactogen regimes of series III the DA

[4] As shown by frozen sections of corpora lutea which had been fixed in Helly's fluid and stained by Rossman's (1942) modification of Romeis' Sudan III technique.



DESCRIPTION OF FIGS. 2–5

Unretouched photomicrographs of portions of corpora lutea from different types of rats. ×525.

FIG. 2. Cyclic DA rat. Corpus luteum of the next youngest set, from a young animal on the first day of a diestrous interval in a natural cycle.

FIG. 3. Normal rat (Vanderbilt strain). Corpus luteum of similar age to that in figure 2. The cells tend to be larger and cytoplasmic vacuolation makes the cell boundaries more distinct.

FIG. 4. Corpus luteum of a persistent-estrous DA rat which had been rendered cyclic by progesterone. Formed during the first cycle; removed on the first day of diestrus after the second cycle.

FIG. 5. Corpus luteum of a persistent-estrous DA rat which had experienced 3 cycles under lactogen treatment (case illustrated in fig. 1, G). Removed on the first day of diestrus after the final cycle. Note the increased cytoplasmic vacuolation, the larger size of the cells and their prominent outlines.

corpora lutea were caused to resemble those of normal Vanderbilt rats, not only in histologic structure, but also with respect to certain histochemical reactions.

Determinations of corpus luteum cell volume. The measurement of the cross-sectional areas of corpus luteum cells gave a direct index of their relative volumes in each of the groups of animals (table 1). The data from normally cyclic animals of the DA and Vanderbilt strains confirmed the visual impression that the Vanderbilt lutein cells are larger. The difference between the respective means of sectional area

TABLE 1. DIFFERENCES OF SECTIONAL AREAS OF CORPUS LUTEUM CELLS AMONG CYCLIC VANDERBILT RATS AND DA RATS OF SEVERAL CATEGORIES.

Group	Number of Animals (N)	Average Area[3]		t	P
		Range	Mean ± S.E.		
		μ^2	μ^2		
DA (cyclic)	4	106–117	112 ± 9.7	4.92	<0.01
Vanderbilt	5	128–146	134 ± 7.2		
				1.62	>0.1
DA (lactogen)[1]	6	125–135	128 ± 3.9		
DA (cyclic)	4	106–117	112 ± 9.7		
				1.18	>0.2
DA (progesterone)[2]	9	95–115	108 ± 6.3		
				7.15	<0.01
DA (lactogen)	6	125–135	128 ± 3.9		

[1] Persistent-estrous rats, cycles maintained by lactogen.
[2] Persistent-estrous rats, cycles maintained by progesterone.
[3] 150 cells measured for each animal and averaged. Each average treated as a single determination.

($112\mu^2$ and $134\mu^2$) was highly significant. The mean area of cells from persistent-estrous DA rats (after a progesterone cycle) was almost identical with that obtained from cyclic DA rats. On the other hand, data from 6 DA persistent-estrous animals which had been carried through at least one "lactogen" cycle (series I, III) gave a mean sectional area ($128\mu^2$) which was significantly greater than the value for cells from the DA 'progesterone cycle' group ($108\mu^2$) and was probably not significantly less than the mean for cyclic Vanderbilt corpus luteum cells.

It may be concluded that lutein cells of cyclic Vanderbilt rats are larger than those of DA cyclic rats, that the cells of the latter are similar in volume to the cells induced in persistent-estrous DA rats by progesterone, and that lactogen treatment which successfully maintains cycles in otherwise persistent-estrous rats enlarges the luteal cells to a volume comparable to that in cyclic Vanderbilt animals. In addition many vacuolated cells occur like those normally present in the Vanderbilt corpora lutea.

DISCUSSION

Whether in the successful attempts to induce ovulatory cycles by prolactin, the conditions have been simulated which naturally occur in normal rats, cannot as yet be stated with certainty. The experiments merely demonstrate that it is possible for a luteotrophic agent such as prolactin to counteract the mechanisms which cause persistent estrus and allied phenomena. Corpora lutea, in this species, can evidently be maintained at levels of activity considerably below that found in pregnancy and pseudopregnancy, levels which do not interfere with the regular recurrence of estrus and ovulation.

It appears that the induction of new corpus luteum formation in these experiments was brought about indirectly through the mediation of progesterone, formed by previously existing luteal tissue. In the absence of such tissue, none of the lactogen regimes employed was effective in bringing about subsequent ovulation. In Astwood's hypothesis (1941) the action of "luteotrophin" was presumed to be confined chiefly to stimulation of preovulatory secretion of progesterone by the graafian follicles. In the present lactogen experiments, had any considerable amount of progesterone been secreted by the follicles, that in itself should have been sufficient to induce ovulation in the *absence* of older corpora lutea. The possibility has not been completely excluded, of course, that the injections of lactogen were not suitably timed to affect the follicles when they were most responsive. It is also conceivable that larger amounts of lactogen during proestrus might be effective.

The present evidence adds to the several indications previously cited that animals of the DA strain are relatively deficient in secretion of prolactin or of an allied substance with similar properties. On this assumption many of the differences between the two strains can be explained. In the cyclic Vanderbilt animals a moderate level of prolactin secretion may be supposed which results in a low-grade stimulus to the corpora lutea during diestrus. A greater stimulus appearing at the onset of estrus would account for secretion of physiologically effective amounts of progesterone at that time. This in turn would indirectly assist ovulation and luteinization.

There is good reason to regard the progesterone formed at such times as a protective factor which is not necessarily essential to the completion of the cycle. The first corpora lutea formed in a young rat are obviously produced in the absence of earlier corpora. Several clear-cut cases have been observed in persistent-estrous animals, in each of which a long-continued period of vaginal estrus was spontaneously interrupted to be followed by a cycle, new corpora lutea forming in the absence of other lutein tissue. Unless the postulate is wrong that the follicles themselves are incapable of forming progesterone, these spontaneously occurring cycles must be interpreted as

having proceeded at least as far as follicle rupture without the mediation of that substance.

That progesterone may not always guarantee the completion of a full ovarian cycle is indicated by the occasional failures under optimum conditions, in the progesterone series (1943) and in the present lactogen series. It may be supposed that the luteinizing factor is not always available in adequate amounts in these animals. Only when it is adequate will progesterone insure its effective release. This particular point is illuminated by an unpublished series of experiments in which I took advantage of Fiske's (1941) evidence that a regime of continuous light tends to diminish the secretion of luteinizing hormone. Persistent-estrous DA rats were placed in continuous light for about two weeks and attempts were then made to induce ovulation by a single injection of progesterone during proestrus. Nearly all of these cycles (11 among 13) were anovulatory, while similar experiments (Everett, 1943), under normal lighting conditions, had resulted in corpus luteum formation in the majority of cases. Since there is reason to believe that the normally lighted persistent-estrous DA rat is moderately deficient in luteinizing hormone (Everett, 1944), it may be considered that continuous illumination aggravated this condition, with the result that when progesterone was administered the available luteinizing factor was inadequate in most cases.

With regard to the presumed action of lactogen on the corpus luteum cells, there are certain questions which require attention. Corpora lutea in the DA strain, under the stimulus of certain levels of lactogen, were transformed so as to resemble histologically the corpora lutea in the Vanderbilt rats. Thus the average cell-volume was increased and vacuolation of the cells of the older corpora suggests that their lipid content was similar to that found in Vanderbilt animals. It is well recognized that when corpora lutea are fully activated, as in pregnancy and pseudopregnancy, this activity is reflected by a pronounced increase in cell volume. Therefore it seems not unreasonable to look upon the relatively small increase seen in the present experiments as indicative of a low-grade stimulus. The increasing lipid content of the corpora lutea, which appears with the onset of estrus in the normal rat and in lactogen-treated DA rats, apparently reflects, in some way, this moderate activation. This question will be treated at some length in a subsequent report.

With respect to possible fluctuations in the level of luteotrophic stimulation during the cycle in "normal" rats, the present experiments suggest several possibilities. Without influencing the length of the cycle, a maximal stimulation could continue from the time of ovulation into the first day of diestrus, ceasing altogether after that time as in series II (fig. 1, C). However, it is difficult to account in this way for a fall in progesterone secretion during diestrus and a subsequent rise during proestrus (Astwood, 1939). If one postulates that

luteotrophin secretion continues at a constant level throughout the cycle as in series I, a similar difficulty is encountered unless fluctuations in other factors change the threshold for the luteotrophin. It appears most likely that in the usual 4- to 5-day cycle secretion reaches its height during proestrus and possibly early estrus, rapidly falling off to a bare maintenance level during metestrus and early diestrus, in a manner comparable to the fluctuating dose levels employed in series III. One should recall that the lactogen content of the pituitary glands of cyclic female rats is said to be higher during proestrus and estrus than it is in metestrus and diestrus (Reece, 1937). Although the lactogen content of the pituitary gland at any one time is evidently far below the amounts contained in the 15 and 30 I.U. doses used in the present study, it is possible that the apparent discrepancy rests in the relative inefficiency of single injections once in 24 hours.

Certain of the mechanisms suggested here may well be peculiar to those few polyestrous species in which estrous cycles follow each other at rapid pace, each follicular phase telescoping into the imperfect luteal phase of the preceding cycle. Greater similarity to the more usual mammalian cycle would probably be found in the longer "pseudopregnant" cycle of these animals. Pertaining directly to this question are certain experiments in series II, in which a continued, strong stimulation of corpora lutea resulted in a delay of the onset of the next estrus, as a result of two factors: the duration of lactogen treatment, and a fixed latent period following the last injection. This implies that, in the natural pseudopregnancy, stimulation of the corpora lutea may cease about 2 days before the appearance of estrus. It is by no means clear whether corpus luteum secretion would also terminate promptly or whether it would continue at diminishing rate into the ensuing estrus. In some manner, however, the conditions prevailing during pseudopregnancy undoubtedly have an effect upon the fate of the crop of follicles which reach maturity immediately thereafter, for whenever pseudopregnancy has been induced in persistent-estrous rats (Everett, 1939, p. 125; and subsequent observations) by infertile copulation, they have regularly experienced cyclic estrus at the close of the prolonged interval, before returning to the persistent-estrous condition.

The suggestion is offered, then that the prolactin regime employed in series III resembles the "pattern" of lactogen secretion in "normally cyclic" rats and that the regimes of series II approach the conditions characteristic of pseudopregnancy. Intermediate conditions may occasionally prevail, as suggested by the moderately prolonged cycles in series I.

SUMMARY

Prolactin was successfully employed under certain conditions to induce cyclic ovulation and corpus luteum formation in persistent-

estrous rats. In the absence of previously formed lutein tissue it was ineffectual. The evidence indicates that graafian follicles are incapable of secreting progesterone under the stimulus imposed. However, when corpora lutea were first induced by the action of injected progesterone, subsequent administration of lactogen at certain low levels resulted in the completion of additional cycles. In several instances, sequences of 2 or more cycles were produced by suitable manipulation of lactogen dose level.

Certain of the lactogen regimes induced changes in the DA corpora lutea which caused them to resemble those of normal rats (Vanderbilt strain). These changes, as seen in 5 to 6-day old corpora lutea, included a significant increase in cell volume and increased cytoplasmic vacuolation, associated with increased lipid content.

Certain evidence was reviewed which supports an hypothesis that animals of the DA strain are relatively deficient in prolactin output. It is postulated that a higher "potential" for prolactin secretion exists in normal (Vanderbilt) animals. This results in the low-grade maintenance of corpora lutea during diestrus, while at the onset of estrus an increasing stimulus accounts for secretion of physiologically effective quantities of progesterone. In turn, progesterone facilitates the liberation of luteinizing hormone, an effect which is regarded as a protective mechanism which may compensate for environmental or intrinsic factors tending to lower LH output. Defectiveness of such a mechanism in DA animals could account for the ease of inducing 'light estrus' and in part, for the early age of onset of spontaneous persistent estrus in that strain.

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NEW BOOKS

FEMALE ENDOCRINOLOGY. Including Sections on the Male. By Jacob Hoffman, Demonstrator and Pathologist in Gynecology. Jefferson Hospital, Philadelphia. xv+788 pp. 180 figures, including some in color. $10.00. W. B. Saunders Company, Philadelphia and London, 1944.

The present volume has been written with primary emphasis upon clinical aspects of reproductive endocrinology, a field grown so vast as measured in numbers of publications that a comprehensive summary is an absolute necessity if the average physician is to keep even reasonably well informed. This aim alone is adequate justification for such a book. The author, however, has attempted an even more praiseworthy task of presenting the fundamentals of glandular physiology in an account which is at once readable and pertinent to the problems both of the physician and investigator.

The book is divided into three parts, namely, Physiology, Clinic and Laboratory. Part I considers the major physiological aspects of endocrine glands and the phenomena controlled by them. Part II, presents the major clinical endocrinopathies, especially as they bear upon the reproductive system. The third and final section describes the laboratory procedures commonly used in endocrine diagnosis, and includes a chapter dealing with the various endocrine preparations which are commercially available.

The text is well balanced and avoids successfully most controversial topics. The reviewer confesses to an unfortunate inclination to compare the physiological sections of the book with the masterly accounts of the same topics which are to be found in *Sex and Internal Secretions*. However, since experimental endocrinology is regarded here as only an adjunct to the clinical sections, it is significant that the only basis for comparison should be a *chef d'oeuvre* of reproductive physiology.

The majority of the illustrations are well selected and informative, and the reproductions are uniformly good. Occasional figures occur, however, which mar greatly the excellent impression created by the majority of them. For example, Figure 3 purports to be a normal developing ovarian follicle, yet the hypertrophied theca interna, the presence of leucocytes in the follicular antrum and the misshapen ovum all betray it as a follicle undergoing frank atresia. One also has the feeling that a more satisfactory photomicrograph of the pituitary body than that presented in Figure 36 could have been secured. And, while on the subject, attention might be called to the curiously shaped object in the center of Figure 38 which is a better caricature of an end man in a minstrel show than a representation of the anterior pituitary gland.

Indeed, it is in the construction of the series of diagrammatic illustrations that one can find the principal grounds for criticism. Not only are errors of fact and doubtful opinions abundantly present in these diagrams, but attention is forcibly drawn to them because of the use of colored reproductions. In the frontispiece, the corpus luteum is stated to have a quiescent effect upon myometrial contractions, yet the figure demonstrates an increased

height of contraction. One also gains the impression that while the follicle regulates anterior pituitary activity, the corpus luteum does not. This is not in accord with most endocrinological opinion, nor with the author's statement that " . . . progesterone inhibits ovulation indirectly by suppressing the hypophyseal sex principles, and not through any direct effect on the follicle" (p. 51). Figure 38 indicates a parathyrotropic and a pancreatropic effect of the anterior pituitary, although both effects are, at least, debatable. These unfortunate instances greatly detract from the excellent impression created by the volume, and should be corrected in later editions.

An extensive bibliography is included at the end of each chapter, and a very useful bibliographic and subject index is presented at the end of the book.

The author's familiarity with the European literature of his subject is an especially valuable aspect of the volume, especially in view of the well organized bibliography. The juxtaposition of laboratory and clinical data serves a very real function of demonstrating the fundamental interdependence of the two aspects of reproductive physiology. The chapters on diagnostic aids and blood and urine findings should aid in disseminating information of the practical techniques of endocrinology. The volume should find a useful place on the shelves of laboratory and office alike.

EDWARD W. DEMPSEY

ASSOCIATION ANNOUNCEMENT

Nominations for Awards of the Association

Two awards for meritorious work in endocrinology will be given at the next annual meeting of the Association. The work may be either clinical or in the basic sciences. A special Committee of five members of the Association chooses the recipients of these Awards, subject to ratification by the Council.

Each member of the Association has the privilege of making one nomination for each Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions in endocrinology and by a bibliography of his most important papers with reprints if possible. Five copies should be sent to the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, *not later than February 15, 1945.* The Awards are described below.

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb and Sons Award of $1,000.00 was established in 1939. It was given in 1940 to Dr. George W. Corner, in 1941 to Dr. Philip E. Smith, in 1942 to Dr. Fred C. Koch, and in 1944 to Dr. Edward A. Doisy. No age or other special limitation is stipulated by the Donor of the Award. So far it has been given for long-continued work of a high quality.

THE CIBA AWARD

The Ciba Award was established in 1942 and is to be given to an investigator not over 35 years of age. No recipient was selected in 1942. In 1944 the Award was given to Dr. E. B. Astwood. The Award is for $1,200.00. If the recipient chooses to use the Award to aid in working in a laboratory other than the one in which he normally is located, the Award will be increased to $1,800.00.

CARL R. MOORE, *President*
HENRY H. TURNER, *Secretary*

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